ACTIVATION, CHARACTERIZATION, INHIBITION AND SEASONAL VARIATION IN THE PHENOLOXIDASE LEVEL OF FLORIDA SPINY LOBSTER (*Panulirus argus*)

By

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Abstract of Dissertation Presented to the Graduate School of
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By

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Major Department: Food Science and Human Nutrition

Phenoloxidase (PO) was found in spiny lobster exoskeleton as inert (IPO1 and IPO2) and/or active (EAPO) forms depending on the molting stage of the lobster. The IPO1 became activated during electrophoresis. The inert PO's were activated by trypsin, producing two active PO forms (TAPO1 and TAPO2) with up to 120-fold increased PO activity. Phenoloxidase catalyzes the oxidation of phenolic compounds to melanin which leads to the darkening of many light fruits, vegetables and crustaceans. Elastase, chymotrypsin, Triton X-100 and repeated freezing and thawing failed to activate PO. The IPO1 became active during storage of a partially purified extract at 4°C; however, only one active PO form (EAPO) was demonstrated by electrophoresis. This activation was prevented by the addition of phenylmethyl sulfonyl fluoride and other protease inhibitors. The inert PO's (IPO1 and IPO2) and a crude PO extract stored at 4°C also became activated, producing only one active PO form. Optimum pH of IPO1 and TAPO2 forms was neutral, while the EAPO was approximately pH 8.0. The IPO1 was most stable at pH 10.0-12.0. The TAPO2 was stable from pH 7.0 to 9.0 while the EAPO had the widest pH stability ranging from 6.0 to 10.0. For all PO forms, little activity was detected below pH 4.0. Apparent Michaelis constants were 0.81, 0.36 and 0.92 mM for IPO1, TAPO2 and EAPO, respectively. Energy of activation values were 12,190; 7,840; and 10,280 cal/mole also for IPO1, TAPO2 and EAPO, respectively. Bisulfite inhibited the melanosis reactions by reacting with intermediate quinones probably forming sulfoquinones and by irreversibly inhibiting PO. Activation of the inert PO's *in vivo* appears to be related to the molting cycle of the lobster. High levels of active PO were found in lobsters ready to molt (late premolt stage) while high levels of inert PO's and low levels of active PO were found in lobsters at the intermolt and premolt stages. Low levels of both inert and active PO's were found in newly molted and postmolt lobsters. Phenoloxidase in the exuvia (cast-off cuticle) was the EAPO. It appeared that PO levels accumulated during intermolt and early premolt as inert forms which became activated when needed for sclerotization

of the newly forming cuticle or any other function (stress) associated with PO utilization.

INTRODUCTION

There are four major deteriorative changes limiting the shelf-life of Florida spiny lobster tails during iced storage. These are spoilage by microorganisms (microbial changes), off-flavor formation (organoleptic changes) due to production of ammonia products (alkyl amines), enzymatic digestion around the gut (textural changes), and discoloration of epidermis and tissues (melanotic changes). Discoloration is one of the most important changes during iced storage. It is known to occur within two to five days on iced storage and is commonly called "black spot" (melanosis). Melanosis connotes spoilage by consumers and is produced by the oxidation of phenolic compounds by the enzyme phenoloxidase. Sulfiting agents still remain the primary treatment for preventing melanosis in shrimp and lobsters. Recently the use of bisulfite has been of great concern because of its ability to induce asthma and other adverse reactions in certain sensitive individuals. Ingestion of sulfiting agents has been linked to several deaths. The mode of action of sulfites in foods is still unknown. In addition, little information on the enzymatic discoloration in crustaceans, specifically Florida spiny lobsters, is available. The spiny lobster industry in the

State of Florida is a $20-million industry. Of the seafood harvested in Florida, lobsters are one of the highest valued commodities; therefore, consumers are more aware of quality changes in this commodity compared to other less-valued products. Studies were initiated to provide more insight into the phenoloxidase system and its inhibition.

Objectives of this study were to study the kinetics of the phenoloxidase catalyzed reaction and the mechanism of activation, to investigate the effect(s) of sulfite on the phenoloxidase system, and to determine the relationship between season and phenoloxidase activity in the Florida spiny lobster.

LITERATURE REVIEW

Two types of browning (enzymatic and non-enzymatic) occur in food systems. Enzymatic browning, melanosis or black spot are general terms which describe the formation of dark quinone polymers (melanins) resulting from phenolic oxidation by phenoloxidases. Phenoloxidases are also known as phenolases, polyphenolases and polyphenoloxidases and will be referred to as phenoloxidases (EC 1.14.18.1) in this study.

Although ubiquitous in nature, phenoloxidases are found mainly in plants; relatively high levels occur in potatoes, mushrooms, apples, peaches, bananas, avocados, tea leaves and coffee (DeMan, 1982). Phenoloxidase does not occur in papaya and presents no problem in kiwifruit due to the lack of substrates. In citrus and other highly acidic foods, the enzyme is lacking or is inhibited by the low pH (Schwimmer, 1981). In general enzymatic browning is deleterious in foods such as lightly colored fruits, but it is desirable for processing of tea and coffee. Phenoloxidase is also present in insects and crustaceans where its main function is in cuticle hardening.

Phenoloxidase catalyzes two types of reactions: hydroxylation of monophenols to give o-diphenols (cresolase activity) and/or oxidation of o-diphenols to yield o-quinones (catecholase activity). It is uncertain whether a single phenoloxidase molecule is responsible for both kinds of activity or if there are truly two different enzyme molecules. Park and Luh (1985) reported cresolase and catecholase activity resulting from different isoenzymes in kiwifruit. Park et al. (1980) detected two isoenzymes in mango that showed activity toward diphenols only. Similar results were obtained by Galeazzi et al. (1981) and by Padrón et al. (1975), working with other fruits. Montgomery and Sgarbieri (1975), working with bananas, found that all isoenzymes encountered exhibited diphenoloxidase activity, however three of these also had monophenoloxidase activity. Thomas and Janave (1986) reported that most of the isoenzymes in bananas showed both monophenoloxidase and diphenoloxidase activity; however, monophenoloxidase activity was not consistently observed in all isozyme forms, especially when the enzymatic extracts were not carefully prepared. They concluded that other researchers failed to find any monophenoloxidase activity because this was inactivated during extraction and purification. Monophenoloxidase has been reported to be of a labile nature (Mayer and Harel, 1979).

Copper is essential for both monophenoloxidase and diphenoloxidase activity (Mason, 1955; Makino et al., 1974) and has been found in phenoloxidases as a prosthetic group (Smith and Krueger, 1962). Phenoloxidases can then be classified as metalloproteins. This characteristic has been used to inhibit the enzyme by adding chelating agents such as EDTA (Mayer, 1962).

The mechanism of oxidation by phenoloxidase on o-diphenols is not clear. The evidence accumulated seems to indicate that oxygen must first be bound by copper, then by diphenol. The mechanism is thought to be ordered Bi-Bi involving two substrates and forming two products (Whitaker, 1972). However, Hamilton (1969) has proposed that three substrates are actually involved, including one O_2 and two diphenols, which form an intermediate perhydroxyl complex. The proposed mechanism is depicted in Figure 1.

The pathway for the formation of melanin from tyrosine (one of the phenoloxidase substrates) proposed by Lerner and Fitzpatrick (1953) is presented in Figure 2. Phenoloxidase catalyzes the first two reactions of the pathway. In the first reaction, phenoloxidase catalyzes the hydroxylation of tyrosine to hydroxytyrosine (DOPA) in the presence of oxygen. This reaction is very slow, but adding small amounts of diphenol helps to increase the rate. In the second reaction, phenoloxidase catalyzes the oxidation of DOPA to dopaquinone by molecular oxygen and this is relatively fast. The reactions following the formation of o-quinone are believed to occur spontaneously. The formation of the red compound dopachrome is important from an analytical perspective in that activity of phenoloxidase is usually determined by measuring dopachrome

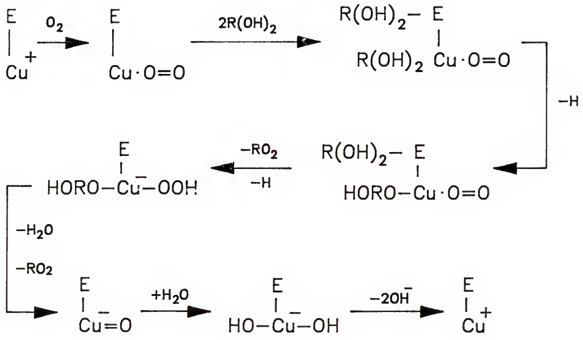
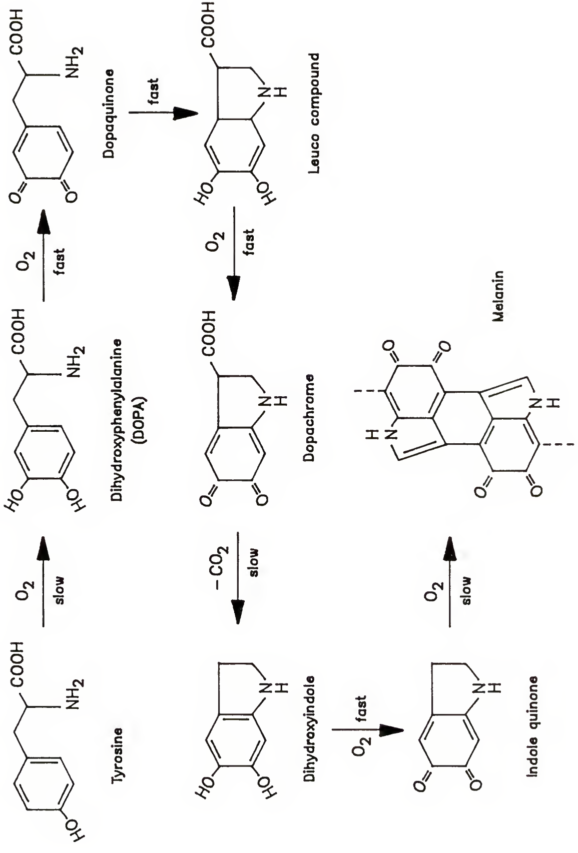


Figure 1. Proposed Mechanism of Oxidation of o-diphenols by Phenoloxidase

Source: Hamilton (1969)

Figure 2. Pathway for the Formation of Melanin

Source: Lerner and Fitzpatrick (1953)


Tyrosine
O2
slow
Dihydroxyphenylalanine (DOPA)
O2
fast
Dopaquinone
fast
Leuco compound
O2
fast
Dopachrome
−CO2
slow
Dihydroxyindole
O2
fast
Indole quinone
O2
slow
Melanin
COOH
NH2
HO
OH
O
N
H

($\varepsilon_{475\ nm} = 3600$) formation spectrophotometrically at 475 nm (Fling et al., 1963). Phenoloxidase activity is also determined by following the formation of melanochrome (purple pigment) at 540 nm (Vachtenheim et al., 1985).

Phenoloxidase in Insects

Studies on different insects have shown the occurrence of multiple forms of phenoloxidases in their blood and cuticle. Blood phenoloxidase shows both mono- and diphenoloxidase activity while cuticle phenoloxidase only shows diphenoloxidase activity (Brunet, 1980). These enzymes are present as inert proenzymes that require an activation process. Most evidence supports the existence of a second enzyme (a protease acting by limited proteolysis) which is required before blood phenoloxidase will function (Brunet, 1980). Price and Hughes (1971) working with *Calliphora* and *Sarcophaga* demonstrated that this natural activator of blood phenoloxidase resides in the cuticle and activates phenoloxidase under the influence of ecdysone (Brunet, 1980). Ecdysone is a hormone that initiates apolysis, a process where the epidermis detaches from the cuticle at the beginning of premolt (Jenkin and Hinton, 1966).

Despite these results, prophenoloxidase has been activated with substances other than enzymes. Sodium dodecyl sulfate (Funatsu and Inaba, 1962) and fatty acids such as palmitic, stearic and linoleic (Heyneman and Vercauteren, 1968) have been reported to activate prophenoloxidase in Musca. Acetone and alcohols have been found to activate phenoloxidase in Lucilia (Preston and Taylor, 1970).

Another physiological aspect of phenoloxidase activation is related to immunity and self-recognition. Prophenoloxidase has been reported to be triggered by microbial products in arthropods. Ratcliffe et al. (1984) demonstrated that microbial products such as laminarin (a β-1,3-glucan extracted from fungal cell walls) not only enhanced phagocytosis but also activated prophenoloxidase present in Galleria blood cells. This activation seems to play an important role in the non-self recognition process. However, Escherichia coli 055:B5 endotoxin failed to activate prophenoloxidase but did enhance phagocytosis in this same system (Ratcliffe et al., 1984).

Pye (1974) found that prophenoloxidase from Galleria was activated with either zymosan (a yeast polysaccharide) or damaged (frozen, rethawed and heated) Pseudomonas aeruginosa in immune plasmas. A high degree of prophenoloxidase activation, similar to that in immune plasmas treated with the microbial products, was shown in both control and immune plasmas after treatment with α-chymotrypsin. Pye hypothesized that zymosan may activate a proteolytic enzyme which would in turn activate prophenoloxidase. He also suggested that the activation may be the result of enzyme aggregation.

Much less research has been performed in the seafood area regarding phenoloxidase activation. Söderhäll and Unestam (1979) activated crayfish serum prophenoloxidase by β-1,3-glucans (carbohydrates) and by purified fungal glycoproteins. They also activated prophenoloxidase using laminaripentaose, an oligosaccharide produced by hydrolysis of a yeast glucan. Söderhäll and Häll (1984) working with crayfish serum prophenoloxidase found that lipopolysaccharides from *Escherichia coli* and *Salmonella abortus* activated a protease which then triggered the activation of prophenoloxidase.

A positive correlation between resistance against *Aphanomyces astaci* (a fungal parasite) and the degree of melanization and phenoloxidase activity in crayfish has been demonstrated by Unestam (1975). This parasite uses proteases and other means (mechanical forces) to penetrate the cuticle of the host (Söderhäll and Unestam, 1975). These proteases may activate prophenoloxidase in the outer layer of the cuticle. Secondly, if these proteases are inactivated by substances in the cuticle, then the extracellular glycoproteins produced by *A. astaci* could still activate prophenoloxidase. Therefore, both activation mechanisms could play an important role in the defense of the host against parasites.

Savagaon and Sreenivasan (1978) have shown that phenoloxidase from shrimp and lobster exists in a latent form which was activated by trypsin or by an endogenous enzyme with tryptic activity found in the tissues. They proposed that the mechanism of activation involved limited proteolysis of the latent phenoloxidase by the activating protease to form isoenzymes. Other proteases such as chymotrypsin and pepsin had no activating effect, although chymotrypsin has been shown to have an activating effect on insect phenoloxidase. Coralase and trypsin have also been shown to activate phenoloxidase in deep sea crab (Marshall et al., 1984, 1985).

The exact mechanism of the activation of phenoloxidase in shrimp and lobsters as well as in insects is still not fully understood. Some similarities in the mode of action of both groups of phenoloxidases should be expected since both lobsters and insects as well as shrimp use phenoloxidases in the sclerotization process. In the process, phenoloxidase oxidizes diphenols to the corresponding quinones, which react with certain side groups on adjacent protein chains, thus linking them together (Stevenson, 1985). N-acetyldopamine (Andersen, 1979) and 3,4 dihydroxybenzoic acid (Pryor et al., 1946) are cross-linking agents that have been identified in crustaceans and in the cockroach, respectively. Hardening of the cuticle may also occur by dehydration (Vincent and Hillerton, 1979). Phenoloxidase is also involved in the process of wound repair of the cuticle and it may also be indirectly involved in the calcification of the cuticle (Stevenson, 1985).

Structure and Composition of the Crustacean Cuticle

The crustacean cuticle has several functions: it protects crustaceans from the environment, it serves as a food storage reservoir, and it forms the exoskeleton which determines the shape of the animal. The cuticle is a composite structure consisting of two main layers, namely the epicuticle and the procuticle (Stevenson, 1985). A schematic view of the crustacean cuticle is presented in Figure 3. The epicuticle is the thin outermost layer subdivided into two layers with different chemical and physical properties, and characterized by the absence of chitin (Andersen, 1979). The principal chemical components of the epicuticle are proteins, lipids and calcium salts. The procuticle is located between the epicuticle and epidermal cells. It constitutes the largest part of the cuticle and contains chitin. The procuticle is a composite structure consisting of the proecdysial layer (secreted before ecdysis) and the postecdysial layer (secreted after ecdysis). The latter is subdivided into the principal layer which is a calcified structure and the membranous layer, uncalcified (Stevenson, 1985). The chemical components of the procuticle are mainly chitin, protein and calcium salts. The membranous layer contains no calcium. Other secondary components are lipids, pigments and small organic molecules (Andersen, 1979).

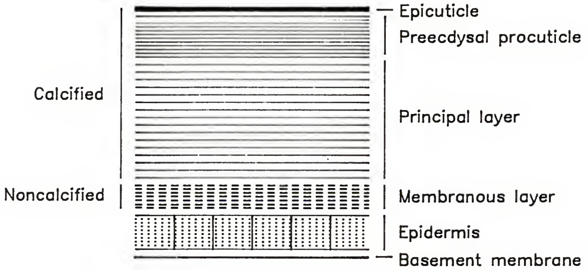


Figure 3. Schematic View of the Crustacean Cuticle

Source: Stevenson (1985)

Molting Stages and their Influence on Phenoloxidase Activity

Crustaceans, insects, and snakes go through a cycle of physiological and morphological events or stages to shed (cast off) their outer exoskeleton, layer, or integument of skin. This process is called the molting cycle or more accurately, ecdysis, and it is divided into four basic periods: postmolt, intermolt, premolt and molt (Aiken, 1980). It has been argued that the terms molt, postmolt intermolt, and premolt are technically inaccurate, and they should be referred to as ecdysis, metaecdysis, anecdysis, and proecdysis, respectively (Stevenson et al., 1968; Skinner, 1985). The sequence of events in the molting cycle with their specific details and characteristics have been summarized by Aiken (1980) and Skinner (1985).

There is a relationship between molting and the build up of melanin precursor, especially in the premolt stage in shrimp (Cobb, 1977). Summers (1967), working with fiddler crabs, reported a significant reduction in the activity of blood phenoloxidase with the onset of premolt. The enzyme activity was lowest in postmolt, but it more than doubled to a maximum activity during intermolt.

Phenoloxidase Inhibitors

Enzymatic browning can be prevented using physical and chemical treatments. These include heat treatment such as blanching of vegetables to inactivate or thermally destroy enzymes, exclusion of molecular oxygen causing substrate limitation, addition of chelating agents to complex the cofactor copper, use of reducing agents to reduce the o-quinones to diphenols preventing polymerization, and use of some enzyme inhibitors. Heat treatment is not used in the seafood industry because it alters the delicate structure of the product (lobster, for example) and consumers are reluctant to buy seafood already cooked, as in the case of boiled shrimp. Oxygen exclusion is usually done in fruits where the oxygen is displaced from the fruit by a sugar solution under vacuum. Addition of chelating agents such as citric acid have been used to inhibit browning in potatoes (Feinberg et al., 1964). Polyphosphates, specifically tripolyphosphate, have been reported to inhibit phenoloxidase in shrimp but at extremely high concentrations (Madero and Finne, 1982). Several other chelating agents including EDTA (Mayer, 1962), diethyl dithiocarbamate (Antony and Nair, 1975), and phenylthiourea (Madero and Finne, 1982) have inhibited browning, but, of these metal complexing agents, only citric acid and polyphosphates are approved for foods.

The reducing agent ascorbic acid is the most widely used chemical to retard enzymatic browning. As soon as the intermediate quinones are formed they are reduced back to diphenols so they cannot form colored polymers. Discoloration is prevented as long as there is ascorbic acid present. Ingraham (1956) reported ascorbic acid has no direct effect on phenoloxidase activity. However, Golan-Goldhirsh and Whitaker (1984) demonstrated that incubation of ascorbic acid with mushroom phenoloxidase effectively inhibited the enzyme. Dithiothreitol and reduced glutathione have been shown to act in a similar manner (Golan-Goldhirsh and Whitaker, 1984).

Cysteine and other food compatible thiols have been shown to inhibit enzymatic browning by reacting with the intermediate quinones (Muneta and Walradt, 1968; Antony and Nair, 1975) to form a quinone-cysteine adduct. However, cysteine may also inhibit the reaction by reducing the quinones back to diphenols (Muneta and Walradt, 1968).

The most successfully used phenoloxidase inhibitor has been sulfur dioxide. It can be used as a gas but is most commonly used as the salt forms (sulfite, bisulfite, and metabisulfite). The aqueous solution of these salts are very effective in inhibiting enzymatic browning, especially at low pH, where the SO_2 form predominates according to the following dissociation reactions,

$$H_2SO_3\ (SO_2 + H_2O) \longleftrightarrow H^+ + HSO_3^-; \quad K_1 = 1.72\text{x}10^{-2}$$

$$HSO_3^- \longleftrightarrow SO_3^{-2} + H^+; \quad K_2 = 6.24\text{x}10^{-8}$$

Extensive research has been carried out on these compounds as enzymatic browning inhibitors; however, the mechanism of this inhibition is not completely understood. Sulfite inhibition of phenoloxidase has been controversial over the years due to failure in realizing that the observed effect is dependent upon the assay method (Golan-Goldhirsh and Whitaker, 1984). Most inhibition studies have been done in fruits and vegetables; mushroom tyrosinase has been the enzyme most examined. Inhibition studies on enzymatic browning using mushroom tyrosinase have shown that sulfur dioxide has more than one mode of action. It both inactivates the enzyme(s) and also inhibits the formation of colored oxidation products by reacting with intermediate compounds (Haisman, 1974; Embs and Markakis, 1965; Golan-Goldhirsh and Whitaker, 1984).

It has been proposed (Schroeter, 1966; Friedman and Gumbmann, 1986) that sulfurous acid salts react with the disulfide bonds of cystine residues in proteins (e.g. soy flour proteins) by a reversible reaction to yield fragments of the original proteins as shown below,

$\text{P-CH}_2\text{-S-S-CH}_2\text{-P'} + \text{SO}_3^{-2} \longleftarrow\!\!\!\longrightarrow \text{P-CH}_2\text{-S}^- + \text{P'-CH}_2\text{-S-SO}_3^-$

The S-sulfocysteine derivative ($\text{P'-CH}_2\text{-S-SO}_3^-$) can interact further with the thiol anion ($\text{P-CH}_2\text{-S}^-$) to regenerate SO_3^{-2} (reversible reaction), so the net effect is a rearrangement of disulfide bonds and regeneration of most sulfite salt. However, this reaction has not been detected in the inhibition of phenoloxidase by sulfurous salts (Sayavedra-Soto and Montgomery, 1986).

Although sulfiting agents are widely used in the seafood industry, very little fundamental research has been done in the seafood area on this inhibition. Several different theories have been postulated on the mechanism of melanosis inhibition in shrimp, including copper sequestering ability, bactericidal ability and reducing properties (Bailey and Fieger, 1954). It has also been suggested that sulfiting agents inactivate the enzyme rather than interfering with the reaction. Reduction of pH has been shown to reduce black spot formation (Faulkner et al., 1954). Madero (1982) has proposed that bisulfite is a competitive inhibitor of the reaction. He suggested that bisulfite appears to bind to a sulfhydryl group located at the active site of the enzyme. To these theories, the following possibilities can also be added for the inhibitory action of bisulfite: reducing oxygen thus making it unavailable for oxidation, reacting with quinones or other intermediates in polyphenol oxidation, reacting with the substrate, and/or reacting with the phenoloxidase activator in the case of insects and crustaceans.

PHENOLOXIDASE FROM THE SHELL OF FLORIDA SPINY LOBSTER: MODE OF ACTIVATION AND CHARACTERIZATION

Introduction

Florida spiny lobsters during storage at refrigeration temperature are susceptible to deteriorative reactions that lower the quality of the product and affect consumer acceptance. One of the most important reactions affecting the appearance of lobsters is melanosis. Melanosis is a discoloration that is due to oxidation of phenolic compounds by phenoloxidase (EC 1.14.18.1). Phenoloxidase may be present as an inert proenzyme that requires activation (Savagaon and Sreenivasan, 1978; Brunet, 1980; Söderhäll and Häll, 1984). The exact mechanism of this activation is not completely understood. Little information has been reported on phenoloxidase in crustaceans while an abundance of information has been published on insects (Brunet, 1980; Andersen, 1979). Some similarities in the mode of action should be expected since both crustaceans and insects use phenoloxidase in the sclerotization process. Understanding the mechanism of enzyme activation not only supplies fundamental information, but also could aid in finding other ways of controlling melanosis as well.

Materials and Methods

Florida lobsters were obtained from Whitney Marine Laboratory at Marineland, FL. The animals were maintained in tanks with sea water circulation. Only tail shell was used for phenoloxidase (PO) analysis. Lobster tails were frozen and transported in ice from Marineland to Gainesville (a 1.75-hr trip). Pleopods and epidermis were not included in the analysis.

The following reagents were used to prepare the buffers for determinations of pH optima: sodium citrate, sodium phosphate (Fisher Scientific Company) and glycine (Sigma Chemical Company).

Phenoloxidase Extraction

Phenoloxidase was prepared by homogenizing in a Waring Blender 1 part lobster shell (by weight) with 4 parts (by volume) 0.05 M potassium phosphate buffer (pH 7.2) for 2 min at 4°C. The homogenate was centrifuged at 10,000g at 4°C for 20 min and the supernatant stored at -20°C until needed.

Determination of Phenoloxidase Activity

Phenoloxidase activity was determined by mixing 0.1 ml of the enzymatic preparation with 2.8 ml of 10 mM DL-β-3,4-dihydroxyphenylalanine, DL-DOPA, (substrate) in 0.05 M phosphate buffer (pH 6.5) with and without trypsin at 23°C (Savagaon and Sreenivasan, 1978). An LKB Model 4050 Ultrospec spectrophotometer interfaced to an Apple IIe computer was used to determine the initial rate of increase in absorbance at 475 nm. Absorbance values were measured for 5 min and the steepest slope calculated by the computer. Phenoloxidase activity was defined as µmoles of DL-DOPA transformed per min at 475 nm at 23°C and under the other conditions specified above. The absortivity of dopachrome at 475 nm has been reported to be 3600 (Fling et al., 1963). Since one molecule of dopachrome results for every two molecules of dopaquinone formed (Fling et al., 1963), 1.0 µmole of DOPA will produce 0.5 µmole of dopachrome which will produce an absorbance of 0.6 under the specified conditions outlined above (e.g. 23°C, 3 ml of total mixture volume, and 475 nm).

Phenoloxidase Activation Treatments

The initial reaction rate was determined as outlined above in the crude PO extracts in the presence of 0.1 ml of 1% solutions of elastase, chymotrypsin, and trypsin (Sigma Chemical Company). The rate of reaction was also measured incubating the crude PO extracts (0.1 ml) with 0.05 ml of 10% Triton X-100. The effect of repeated freezing and thawing (5 times) on the crude PO preparations was also determined.

Analytical Electrophoresis and Molecular Weight Determination

Analytical nondenaturing polyacrylamide gel electrophoresis (PAGE) was used to test for multiple forms of PO and to aid in understanding the mechanism of its activation. A Bio-Rad Model 3000/300 or Pharmacia power supply was used in the electrophoresis. Gel tubes 0.5 cm

I.D. by 12.5 cm high, containing 5% acrylamide unless otherwise specified were run at a constant current of 3 mA/tube. Sample size was 50 µl and chamber buffer was 0.01 M tris-(hydroxymethyl) amino methane/0.04 M glycine, pH 8.3 (Sigma Chemical Company, 1984).

Molecular weights (MW) of the different PO forms were determined using the Sigma Nondenatured Protein Molecular Weight Marker Kit (Sigma Chemical Company). The procedure is outlined in the Technical Bulletin No. MKR-137 (Sigma Chemical Company, 1984) which is a modification of the methods of Bryan (1977) and Davis (1964). The standard proteins used were bovine milk α-lactalbumin (MW = 14,200), carbonic anhydrase from bovine erythrocytes (MW = 29,000), chicken egg albumin (MW = 45,000), bovine serum albumin (MW = 66,000 and MW = 132,000 for the monomer and dimer, respectively) and jack bean urease (MW = 240,000 and MW = 480,000 for the dimer and tetramer, respectively). Molecular weight determinations were made using a 50 µl sample on each 4, 5, 6, 7, 8 and 9% acrylamide gels.

Preparative Electrophoresis

Preparative nondenaturing polyacrylamide gel electrophoresis (PAGE) was performed using a Bio-Rad Model 175 gel tube chamber attached to either a Bio-Rad Model 3000/300 power supply or to a Pharmacia Model EPS 500/400 power supply. Gel tubes were 1.4 cm I.D. by 12 cm long and had 14 ml of the running gel mixture (5% acrylamide) poured into each tube. Sample size of 1.0 ml was applied to the gels which then were run at a constant current of 8 mA/tube.

Nondenaturing PAGE was conducted on the PO preparations with trypsin, without trypsin, and on the preparations activated during storage at 4°C. A 5 mM DL-DOPA solution in 0.05 M phosphate buffer (pH 6.5) was used as a specific enzymatic staining agent for phenoloxidase bands either in the presence or absence of trypsin. Two out of eight gels were stained in this manner and the relative migrations (R_f's) calculated for the bands showing PO activity. The PO forms were isolated from the other six gels by slicing the polyacrylamide gel at the R_f band and extracting with 10 ml of 0.05 M phosphate buffer pH 7.2 using a glass tissue grinder. The homogenate was centrifuged at 10,000g for 15 min at 4°C and the supernatant saved for PO analysis. The top band (IPO1) was separated in the same manner, activated and then rerun electrophoretically.

Fast Protein Liquid Chromatography

Fast protein liquid chromatography (FPLC) was also used to separate multiple forms of phenoloxidase using a Superose 12 column (1 cm I.D. by 30 cm long) with a molecular weight exclusion limit of 4×10^7 (Pharmacia). The FPLC was performed with a high performance liquid chromatography (HPLC) system integrating a Waters Associates Model 6000A pump, a Waters Model U6K injector, and an ultraviolet detector at 280 nm. Volume of sample injected was 0.2 ml; flow rate of the 0.05 M phosphate buffer (pH 7.2) eluent was

1 ml/min. A fraction collector (Gilson Micro Fractionator) was used and the fractions collected were tested for PO activity as described earlier.

Storage Study

A crude PO extract was prepared as previously outlined and stored at 4°C in a refrigerator for five days. The PO activity was measured every day in the absence of trypsin, however trypsin was used to measure PO activity on day one. Every other day analytical nondenaturing electrophoresis was performed on an aliquot as previously described. Two separate replicates were conducted.

pH Optima

The stock buffers were 0.1 M and had the following pH's: citrate buffer (pH 2, 3, 4 and 5), phosphate buffer (pH 6, 6.5, 7.0, 7.5 and 12) and glycine (pH 9 and 10). A 10 mM DL-DOPA solution was prepared in distilled water and an equal volume (1.4 ml) of this solution was mixed with the desired buffer solution to give a 5 mM DL-DOPA in 0.05 M of the respective buffer. To this mixture 0.1 ml of the appropriate PO preparation was added and the initial rate of reaction recorded using a Gilford Instruments spectrophotometer Model 250. Three replicates of the complete experiment were conducted.

pH Stability

Reagents and buffers used for pH stability were similar to those used for pH optima. A volume (0.1 ml) of the PO preparation in deionized and distilled water was mixed with

0.3 ml of the appropriate buffer and the mixture incubated for 30 min in an ice bath. Then, 2.6 ml of 5 mM DL-DOPA in 0.05 M phosphate buffer (pH 6.5) was added and the initial reaction rate measured at 475 nm using a Gilford Model 250 spectrophotometer. The complete experiment was replicated twice.

Temperature Stability

Aliquots of 0.5 ml of the purified PO preparation were heated in a recirculating water bath with controlled temperature for 30 min at 20, 25, 30, 35, 40, 45, 50, 55 and 60°C. The samples were then placed in ice for 5-15 min and PO activity determined as outlined earlier. A volume of 0.2 ml of the heated preparation was used for the activity determination at ambient temperature (23°C). Two replicates of the complete experiment were conducted.

Energy of Activation

Phenoloxidase activity was determined at 20, 25, 30, 35, 40, 45 and 50°C using 2.8 ml of 10 mM DL-DOPA in 0.05 M phosphate buffer (pH 6.5) and 0.2 ml of PO preparation. The initial rate of the reaction was measured at 475 nm using a Beckman DU 8 spectrophotometer with temperature control equipped with kinetics software package. Three replicates of the complete experiment were conducted.

Michaelis Constants

Michaelis constants (Km) were determined from the Lineweaver-Burk reciprocal plots (Lehninger, 1975). Phenoloxidase activity was determined at 23°C using various

DL-DOPA concentrations in 0.05 M phosphate buffer (pH 6.5) ranging from 0 to 10 mM. Initial reaction rates were measured using a Gilford Model 250 spectrophotometer at 475 nm as stated earlier. The complete experiment was replicated three times.

Results and Discussion

The specific activity of the PO extracts varied widely from lobster to lobster. Specific activity values from 0.01 to 0.79 μmole min^{-1} mg protein^{-1} for free activity and from 0.32 to 37.00 μmole min^{-1} mg protein^{-1} for trypsin-activated extracts were found (26 specimens analyzed). The nature of this wide variation was unknown at this point, but will be discussed in a later chapter.

Centrifugation studies suggested that lobster shell PO was soluble since most of the enzyme activity was present in the supernatant after centrifugation for 30 min at 30,000g (Table 1). However, this was not sufficient to sediment light membranes. The crude extract was very stable at -20^{o}C and could be stored at this temperature for several weeks.

Activation of Lobster Cuticle Phenoloxidase

Some PO extracts from crude shell had initially very low PO activity; however, a gradual increase in PO activity occurred during storage at 4^{o}C with the highest activity on the third day, followed by a decline (Figure 4). This was true for some of the shell extracts while other extracts did not demonstrate this same type of trend (data not shown).

Table 1

Phenoloxidase Activity, µmoles DL-DOPA/min/0.1 ml Crude Extract, in the Supernatant, Before and After Centrifugation at 30,000g for 30 min

	NON-CENTRIFUGED	CENTRIFUGED
Free PO	0.014	0.011
Trypsin-activated PO	0.124	0.116

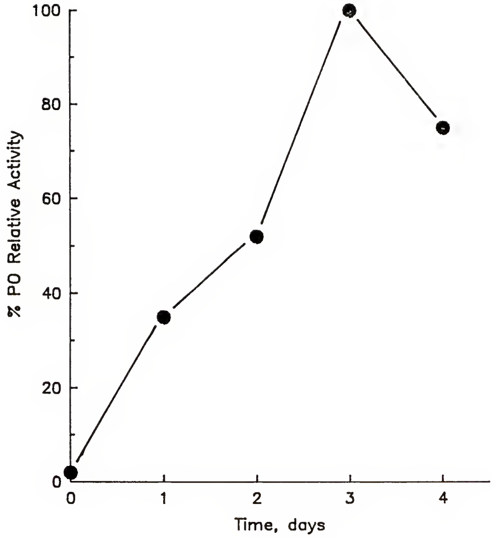


Figure 4. Effect of Storage on PO Activity

Nondenaturing PAGE at day 0 showed the occurrence of one major form of PO at the top of the electrophoretic gel after DOPA staining (Figure 5). This band having PO activity migrated barely into the gel even at gel concentrations as low as 3%. This PO form will be referred as IPO1 (inert PO1). The electrophoretic pattern varied from lobster to lobster but in general the major band was located at the top of the gel, especially for shell extracts with low PO activity.

Nondenaturing PAGE was also performed on the extracts at days 1, 3 and 5 (Figure 5). After staining the gels with 5 mM DOPA, a new band developed at R_f 0.48, the top band gradually disappeared, and was practically gone by day 5. It was thought that the new band was an active form of PO derived from the larger PO (inert) at the top of the gel, and probably produced by the action of an "endogenous protease" present in the crude extract. The presence of an "endogenous protease" in similarly prepared extracts of lobster shell has been demonstrated by Savagaon and Sreenivasan (1978).

To test for the PO activity of the top band (IPO1), a sample of crude extract containing IPO1 was electrophoretically run as before. The top band was extracted by slicing the first 2 mm from the top of the gel, homogenizing with 0.05 M phosphate buffer pH 7.2 using a glass tissue grinder, and centrifuging at 10,000g for 20 min to separate the solid gel. Gels were not stained with DOPA.

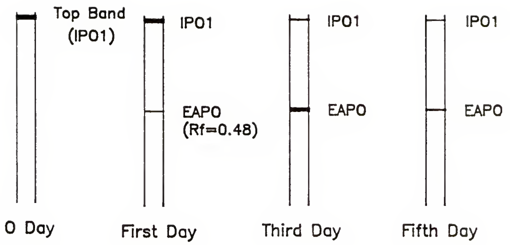


Figure 5. Formation of the Endogenously Activated PO Form (EAPO) as Demonstrated by Nondenaturing PAGE (Gels are 5% Acrylamide)

The supernatant was tested for PO activity and found to be 15 times higher in activity than the crude extract. No explanation was found for this activation at this time, however the presence of a protease inhibitor in the crude extract being removed by electrophoresis was speculated. When the extract from the gel was combined with crude extract, no decrease in the PO activity occurred, indicating an inhibitor was not present. Activation of this PO form may be due to its denaturation by electrophoresis.

It was also observed that the activity of the extracts from the top of the gel increased with time and storage at refrigeration or at freezing temperatures, although, this increase was less noticeable. Electrophoretic analysis of this extract after 24 hr storage (at $4^{o}C$) showed a single highly active PO band was formed with R_f 0.47 which was similar to PO from the storage study of the crude extract. The top PO band may be an inert phenoloxidase form (IPO1) which becomes activated. To confirm the nature of this activation the following inhibition study was conducted.

Inhibition study

After electrophoresis of the non-activated PO preparation, the upper band (IPO1) was separated from the gel by slicing the top 2 mm of the gel, homogenizing in a glass tissue grinder with 0.05 M phosphate buffer (pH 7.2), and centrifuging (10,000g) at $4^{o}C$. The supernatant was divided into several equal volume aliquots, 1 to 10, and treated as follows, Aliquots 1 and 2: No treatment. Stored for 24 hr at refrigeration and freezing temperatures, respectively.

Aliquots 3 and 4: Stored for 24 hr at refrigeration and freezing temperatures, respectively, in the presence of 0.2% trypsin inhibitor.

Aliquots 5 and 6: Stored for 24 hr at refrigeration and freezing temperatures, respectively, in the presence of 0.2% aprotinin.

Aliquots 7 and 8: Stored for 24 hr at refrigeration and freezing temperatures, respectively, in the presence of 2% 2-propanol.

Aliquots 9 and 10: Stored for 24 hr at refrigeration and freezing temperatures, respectively, in an environment saturated with phenylmethyl sulfonyl fluoride (PMSF).

The results presented in Table 2 showed all the protease inhibitors prevented the activation of the IPO1 to some degree. The most effective inhibitor was PMSF, especially at freezing temperature where a 100% inhibition occurred. Aprotinin and trypsin inhibitor also worked very well at freezing temperature with 81.8 and 69.7% inhibition, respectively. A 2% 2-propanol treatment was carried out because PMSF was dissolved in this solvent. Storing the IPO1 preparation at freezing temperature alone caused over 50% inhibition in PO activation. It appeared that the activation of IPO1 under these conditions was of enzymatic nature since protease inhibitors can retard the activation of PO. Controls carried out showed that 2-propanol, trypsin

Table 2

Effect of Protease Inhibitors on Phenoloxidase Activation

	PERCENT INHIBITION[*]	
TREATMENT	Refrigeration temperature	Freezing temperature
No trt., 1 day	0.0	43.2 ± 8.6
Propanol	21.7 ± 1.5	69.8 ± 6.2
0.2% TI[**]	23.5 ± 3.0	64.1 ± 4.7
0.2% aprotinin	57.6 ± 5.3	87.9 ± 7.3
Sat. PMSF[***]	86.6 ± 3.1	99.1 ± 1.5

[*] % inhibition ± standard deviation

[**] Soybean trypsin inhibitor

[***] Saturating concentration of phenylmethyl sulfonyl fluoride

inhibitor, aprotinin and PMSF had no effect on the conversion of DL-DOPA to dopachrome. Incubation of 0.1 ml of the partially purified IP01 preparation for 48 hr with 2.8 ml of DL-benzoyl arginine p-nitroanilide hydrochloride (BAPNA) resulted in the development of a yellow color, characteristic of trypsin or "trypsin-like" proteases while a simultaneous control (no enzyme) showed no color development.

Effect of Proteases and other Treatments on Activation of Inert Phenoloxidase

Phenoloxidase was also activated by trypsin, a serine protease. The presence of 0.1 ml of 1% trypsin solution in a mixture of 2.8 ml of DL-DOPA and 0.1 ml of crude inert PO extract readily increased the phenoloxidase activity from 2-fold to over 120-fold in some cases. Under these testing conditions a lag appeared at the beginning of the reaction and then the PO activity increased gradually until it reached a maximum by 2.5 min. The steepest slope was used to calculate reaction rate. Phenoloxidase activity increased with concentration of trypsin up to 1 mg trypsin/3 ml total reaction volume and then levelled off (Figure 6). Other serine proteases, chymotrypsin and elastase, did not activate the enzyme indicating a very specific action of trypsin on inert PO. Triton X-100 and repeated freezing and thawing also failed to activate lobster shell phenoloxidase (Table 3).

Nondenaturing electrophoresis of crude trypsin-activated PO extract showed the occurrence of two bands with

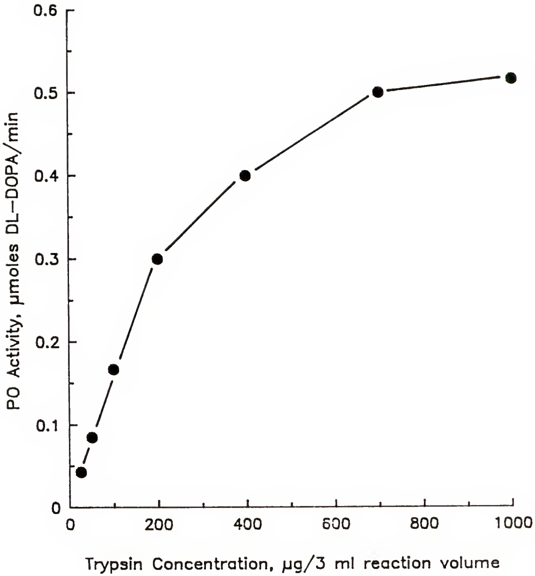


Figure 6. Effect of Trypsin Concentration on the PO Activity of a Crude Extract

Table 3

Effect of Various Proteases, Chemicals and Physical Treatments on the Activation of a Crude Phenoloxidase Preparation

TREATMENT	PO ACTIVITY (µmoles DL-DOPA/min)
No treatment	< 0.003
1% elastase	< 0.003
1% chymotrypsin	< 0.003
10% Triton X-100	< 0.003
Freezing and thawing	< 0.003
1% trypsin	0.528 ± 0.045

PO activity, thus indicating that the mode of action of the endogenous protease appears to be somewhat different than trypsin. It appears that trypsin hydrolyzed IPO1 (the top band) to give two trypsin-activated PO forms (TAPO1 and TAPO2) of lower molecular weights (Figure 7). This was confirmed by trypsin treatment of a partially purified IPO1 solution prepared as for the inhibition study. Two trypsin-activated PO forms (TAPO1 and TAPO2) also were produced from the top IPO1. The TAPO2 seemed to be the dominant trypsin-activated PO form as shown by the greater intensity of this band after developing of the electrophoretic gels (Figure 7) and it will be further characterized. It was concluded that the two active PO forms produced in the crude extract resulted from the action of trypsin on the top band of IPO1.

Fast protein liquid chromatography of the enzymatic extract with and without trypsin added confirmed the possibility of hydrolysis of the IPO1 into two smaller active PO forms. Without any trypsin added, the PO activity was present in the void volume (Figure 8), whereas with trypsin added, the PO activity in the void volume decreased and two new peaks with PO activity emerged (Figure 9).

Occurrence of Additional Phenoloxidase Forms

Nondenaturing analytical electrophoresis of a crude extract was carried out as outlined earlier. The polyacrylamide gels were stained with a 5 mM DL-DOPA solution (pH 6.5) containing 1% trypsin. An additional band developed (referred as IPO2, or inert PO form 2) indicating

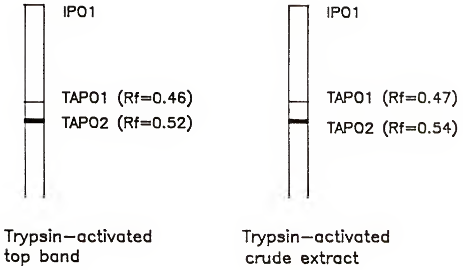


Figure 7. Electrophoresis of Trypsin-Activated Top Band and Trypsin-Activated Crude Extract

Figure 8. A FPLC Chromatogram of a Phenoloxidase Extract Before Addition of Trypsin

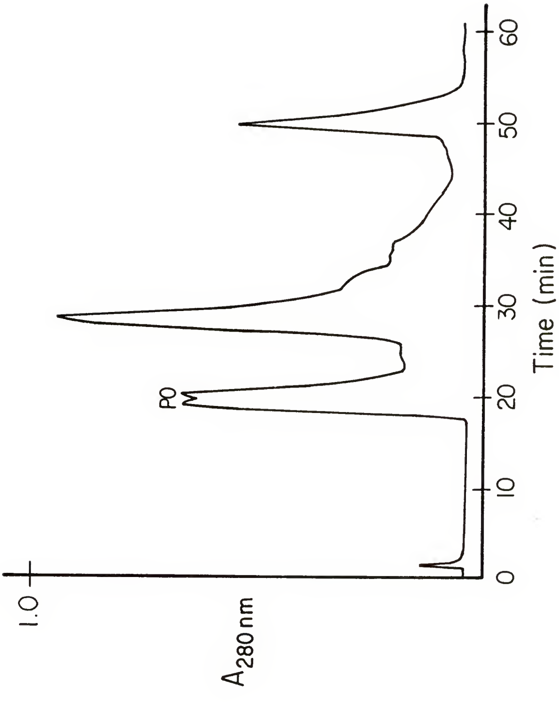
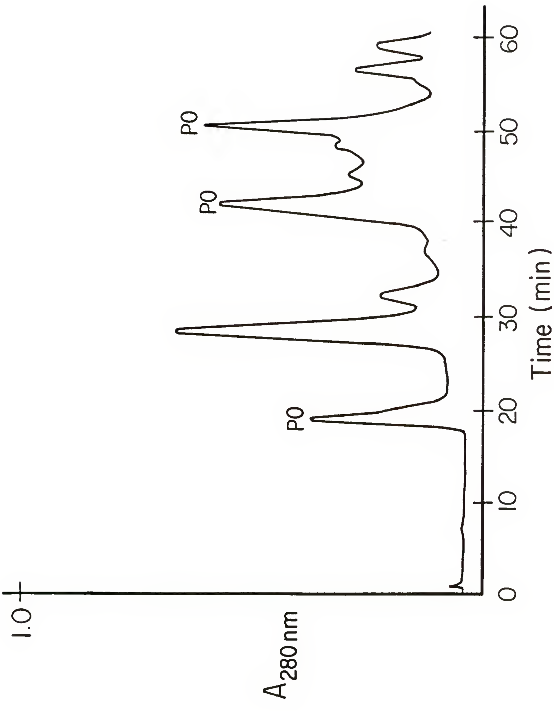

1.0
A280nm
PO
0
10
20
30
40
50
60
Time (min)

Figure 9. A FPLC Chromatogram of a PO Crude Extract After Addition of Trypsin


1.0
A280nm
PO
PO
PO
0
10
20
30
40
50
60
Time (min)

the possibility of more than one inactive form of PO being activated by trypsin (Figure 10). However, when the crude extract was treated with trypsin before electrophoresis, only two bands developed after staining with DL-DOPA as shown in Figures 7 and 10.

Ammonium sulfate fractionation was carried out on an inert PO extract. Most of the inert PO activity was precipitated in the 30-50% ammonium sulfate fraction. Nondenaturing electrophoresis of this partially purified preparation showed the development of new PO bands (Figure 11a). Staining of the gels with a DL-DOPA/trypsin mixture resulted in the development of the second inert PO band (IPO2) with R_f 0.45 (Figure 11b). When the extract was treated with trypsin before electrophoresis, only two bands developed as usual (Figures 7, 10 and 11c). Electrophoresis of this preparation after 3 days at refrigeration temperature showed the occurrence of only one phenoloxidase band as shown in Figure 5.

These results as well as the others seem to indicate that all PO forms, either active or inactive, came from the IPO1 molecule (top of gel). Limited proteolysis of this molecule may have occurred giving rise to either active and/or inactive PO forms. Also, treatment with ammonium sulfate may have disrupted the IPO1 producing different PO forms. Results indicated, there was formation of only one active PO form (EAPO) when the proenzyme was activated by the "endogenous protease" and two active forms (TAPO1 and

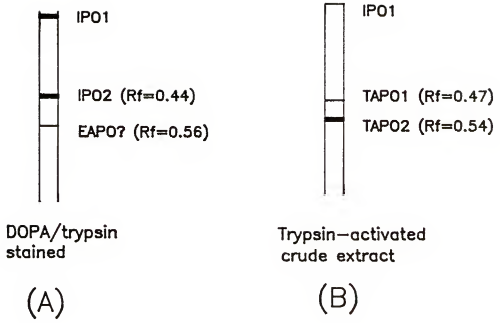


Figure 10. Electrophoresis of an Inert PO Crude Extract Stained with DOPA/Trypsin (A) and a Trypsin-Activated PO Crude Extract (B)

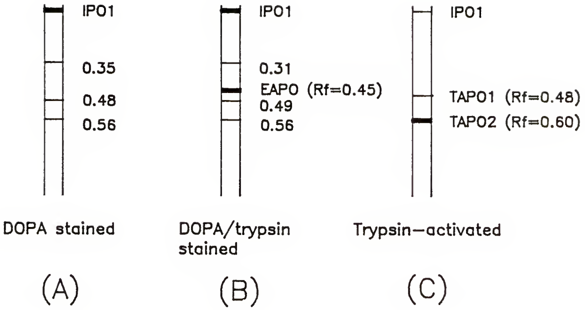


Figure 11. Electrophoresis of a 30-50% Ammonium Sulfate Fraction (nonactivated) using DL-DOPA (A) and DOPA/Trypsin (B) as Specific Staining Solutions, and a Trypsin-Activated Fraction (C)

TAPO2) when activated by trypsin regardless of the number of initial PO forms detected before activation.

pH Optima and pH Stability

Optimum pH of IPO1 and TAPO2 forms was at neutral pH (Figures 12a and 12b), while the endogenously activated form (EAPO) was pH 8.0 (Figure 12c). Optimum pH values of near neutrality have been reported by Benjamin and Montgomery (1973) for PO from Royal Ann cherries, by Satjawatcharaphong et al. (1983) in "Red Delicious" apples, by Cash et al. (1976) in Concord grapes, and by Kahn (1977) in Lerman and Fuerte avocado varieties.

Even though pH 6.5 was not the optimal pH, it was chosen to determine PO activity because DL-DOPA readily darkened at higher pH. Above pH 9, DL-DOPA quickly darkened and there was no possibility for determining the rate of the enzymatic reaction. Even at pH 8, a control having only DL-DOPA (no enzyme) had to be run to determine the contribution of chemical catalysis (darkening of DL-DOPA) on the enzymatic reaction. There was little PO activity below pH 4 and almost none at pH 2.

The different PO forms were very unstable at acidic pH, especially below pH 4.0 (Figure 13) and most of the activity was not recovered when brought back to pH 6.5, indicating irreversible denaturation of the enzyme at low pH's. The IPO1 was most stable at pH 10.0-12.0 as shown in Figure 13a (enzyme activity was not determined above pH 12). The TAPO2

Figure 12. Optimum pH Curves for IPO1 (A), TAPO2 (B) and EAPO (C) Forms

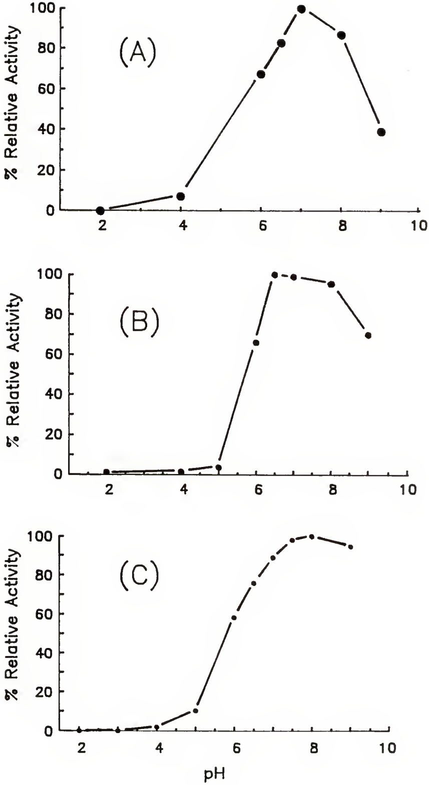
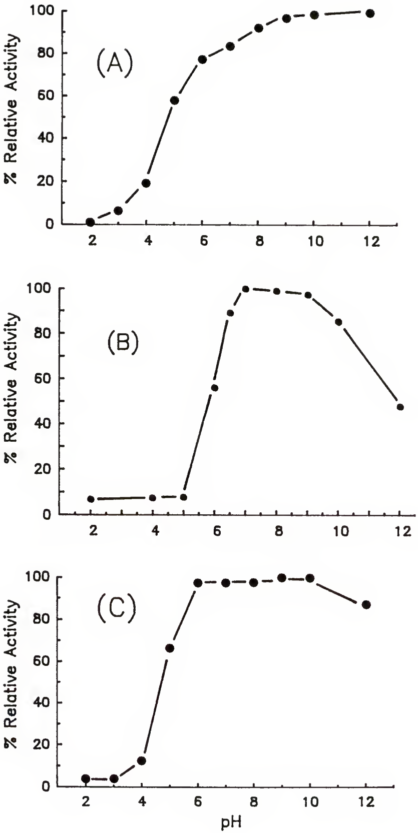

(A)
% Relative Activity
0
20
40
60
80
100
2
4
6
8
10
(B)
% Relative Activity
0
20
40
60
80
100
2
4
6
8
10
(C)
% Relative Activity
0
20
40
60
80
100
2
4
6
8
10
pH

Figure 13. pH Stability Curves for IPO1 (A), TAPO2 (B) and EAPO (C) Forms


(A)
% Relative Activity
0
20
40
60
80
100
2
4
6
8
10
12
(B)
% Relative Activity
0
20
40
60
80
100
2
4
6
8
10
12
(C)
% Relative Activity
0
20
40
60
80
100
2
4
6
8
10
12
pH

was stable from pH 7.0 to 9.0 (Figure 13b) while EAPO had the widest pH stability range of 6.0 to 10.0 (Figure 13c).

The influence of pH on the activity of the PO reaction is not surprising due to the ionic properties of the enzyme and DL-DOPA. The active site of enzymes is usually composed of ionizable amino acid groups that must be in a particular ionic state to maintain the conformation of the site so catalysis can occur. The substrate (DL-DOPA) also contains ionizable groups, namely carboxyl and amino groups which may need to be properly ionized for catalysis.

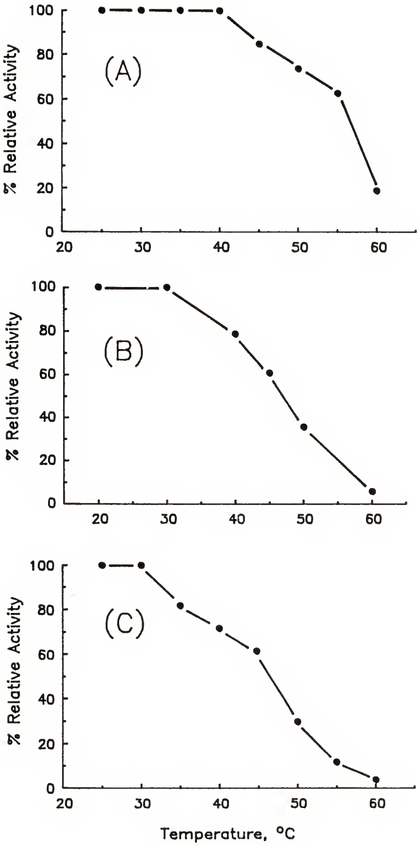
The stability of PO at different pH's must be also taken into account since denaturation of the enzyme can occur at some pH values, thus lowering the enzyme activity. The EAPO was found to be stable in the pH range from 6 to 10; however, the optimum pH was 8.0. Therefore, the decrease in activity below and above pH 8, in the pH range of 6 to 10, was due to the formation of the improper ionic form of EAPO and/or DL-DOPA, rather than enzyme denaturation.

In some cases the pH optimum does not fall in the range of pH where the enzyme is most stable. This is the case for IPO1 which had an optimum pH of 7.0, but was most stable at pH 10-12. Similarly, TAPO2 had an optimum pH equal to 6.5-7.0 and a pH stability range from 7 to 9.

Temperature Stability and Energy of Activation of the Different PO Forms

The EAPO and TAPO forms were stable at temperatures below 35°C, (Figures 14b and 14c) while IPO1 was stable below 45°C (Figure 14a). In all cases, most of the enzyme

Figure 14. Temperature Stability of IPO1 (A), TAPO2 (B) and EAPO (C) Forms


(A)
% Relative Activity
100
80
60
40
20
0
20
30
40
50
60
(B)
% Relative Activity
100
80
60
40
20
0
20
30
40
50
60
(C)
% Relative Activity
100
80
60
40
20
0
20
30
40
50
60
Temperature, °C

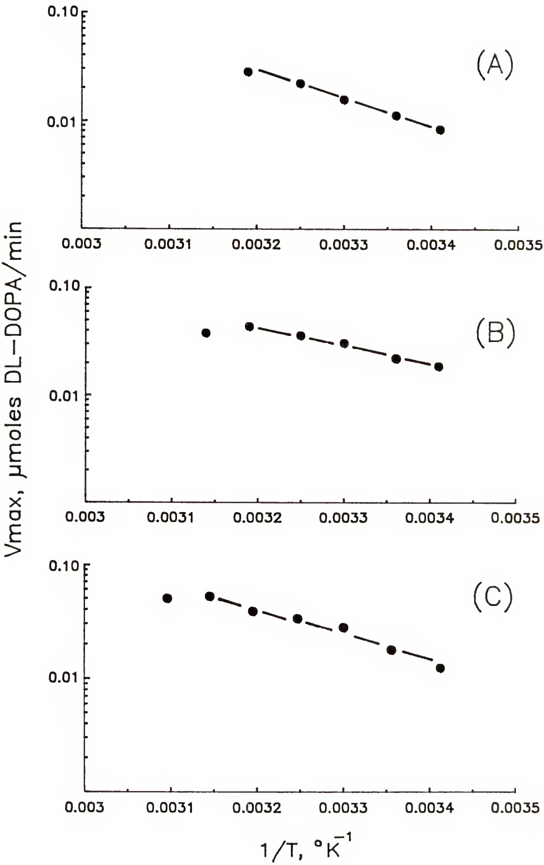
activity was lost at 60°C (Figure 14). Savagaon and Sreenivasan (1975) reported 83-100% inactivation after heating PO preparations from Panulirus homarus Linn. for 15 min at 60°C.

As calculated from the Arrhenius plot, energies of activation (Ea) for IPO1, TAPO2, and EAPO forms were 12,190 ± 950; 7,840 ± 730; and 10,280 ± 820 cal/mole, respectively (Figure 15). In determining these values for Ea, the decrease in the solubility of oxygen occurring at higher temperatures was disregarded. Energy of activation values of 3,000 and 5,200 cal/mole have been reported by Bailey et al. (1960) for PO from shrimp blood. These Ea values were obtained using pyrocatechol as substrate.

For the TAPO2, it was observed that an increase in PO activity occurred up to 40°C. Beyond this temperature, the activity of enzyme started dropping (Figure 15b). The EAPO behaved in a similar way but activity started dropping at 45°C rather than at 40°C (Figure 15c). No decrease in activity was observed for IPO1 up to 40°C (Figure 15a).

It is known that activation energies for enzyme-catalyzed reactions are in the range of 6,000 - 15,000 cal/mole, while activation energies for enzyme denaturation are much higher, in the range of 50,000 to 150,000 cal/mole (Whitaker, 1972). Since an increase in temperature imparts more energy to the reactant molecules, there is a higher chance for these molecules to pass through a transition state by overcoming the minimum energy requirement (energy

Figure 15. Arrhenius Plots for IP01 (A), TAP02 (B) and EAPO (C) Forms


0.10
0.01
(A)
0.003
0.0031
0.0032
0.0033
0.0034
0.0035
0.10
0.01
(B)
0.003
0.0031
0.0032
0.0033
0.0034
0.0035
0.10
0.01
(C)
0.003
0.0031
0.0032
0.0033
0.0034
0.0035
Vmax, µmoles DL–DOPA/min
1/T, °K⁻¹

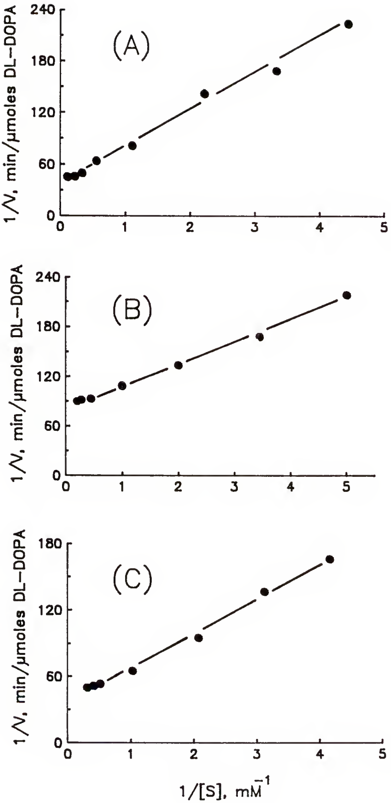
of activation) so that a "fruitful reaction" can occur. But, as the temperature increases, more enzyme molecules attain sufficient energy to reach the denatured state and the reaction rate drops. The lower the energy of activation the easier the reaction occurs, thus the reaction catalyzed by TAPO2 would occur more easily than the reactions catalyzed by the EAPO or the IPO1.

Michaelis Constant (Km)

The numerical values of the apparent Km's were 0.81 ± 0.04; 0.36 ± 0.05 and 0.92 ± 0.08 mM for IPO1, TAPO2, and EAPO forms, respectively, as determined using the Lineweaver-Burk plots (Figure 16). The substrate used was DL-DOPA. None of the three PO forms acted on tyrosine, thus attempts to determine Km values for tyrosine failed. It has been reported (Brunet, 1980) that PO from the cuticle of insects does not present monophenoloxidase activity; however, blood PO does. The diphenols needed for the cuticle PO to form the tanning quinones may be produced in the blood of the insect and transported to the cuticle. A similar mechanism may occur in lobsters.

Michaelis constants of 0.50 mM (substrate was L-DOPA) for blood PO in the fiddler crab (Summers, 1967), 0.67 mM (substrate was catechol) for Concord grape PO (Cash et al., 1976), and 3.7 and 6.0 mM (substrate was DL-DOPA) for Lerman and Fuerte avocado PO, respectively, (Kahn, 1977) have been reported.

Figure 16. Lineweaver-Burk Plots for Km Determination of IPO1 (A), TAPO2 (B) and EAPO (C) Forms


(A)
(B)
(C)
1/V, min/µmoles DL–DOPA
0
60
120
180
240
0
1
2
3
4
5
1/[S], mM^{-1}

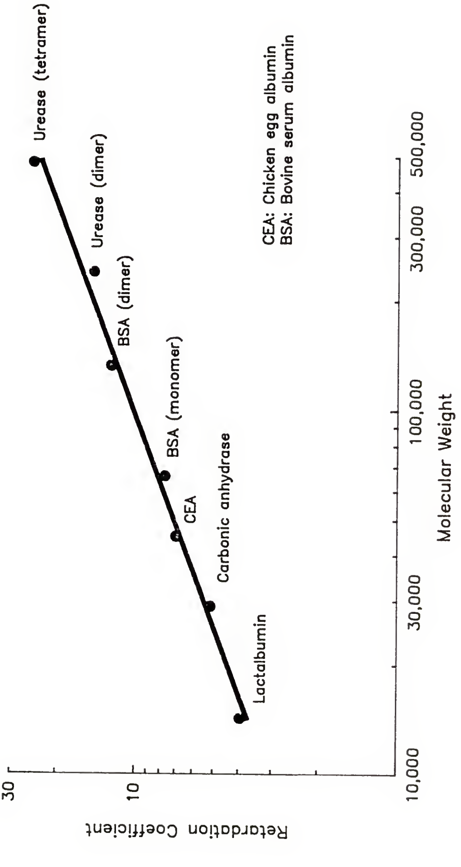
One of the interests of determining Km is because it gives information about the approximate concentration of the substrate for intracellular enzymes in the living cell, and also because it provides information regarding the affinity of the enzyme for the substrate. The oxidation of DOPA by phenoloxidase will lead to melanin; however, there is very little melanin formation in the sclerotization process (Brunet, 1980; Andersen, 1971). It has been proposed that N-acetyldopamine is the major tanning agent involved in the sclerotization of insect cuticle (Andersen, 1971). The N-acetyl group may prevent melanin formation on oxidation. N-acetylamine may be formed by decarboxylation of DOPA by the enzyme DOPA decarboxylase. Also, 3,4-dihydrobenzoic acid has been reported as a tanning agent (Pryor et al., 1946; Pryor, 1962). This compound has been reported to tan the egg-capsule of the cockroach (Pryor et al., 1946). These two cross-linking agents probably are the actual substrates for PO. Their Km values were not determined.

Since lobster tails darken during storage, DOPA may be the substrate for this particular problem. The substrate DOPA may also lead to melanin formation that occurs when the lobster shell is wounded (Lai-Fook, 1966).

Molecular Weight

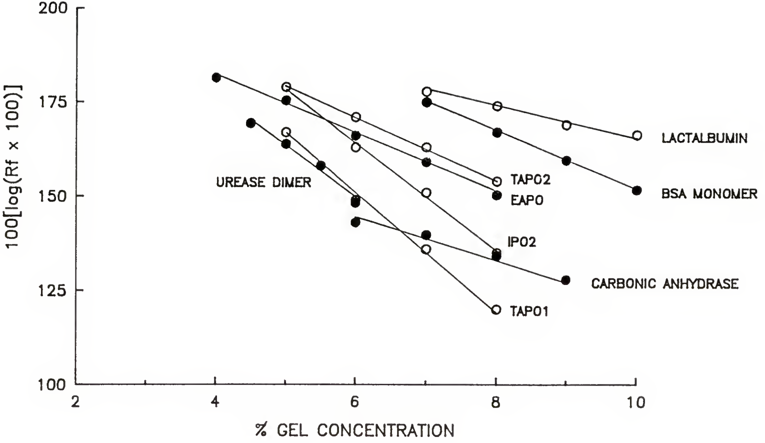
Molecular weights were determined using the Sigma Nondenatured Protein Molecular Weight Marker Kit (Sigma Chemical Company, Bulletin No. MKR-137, 1984). The standard curve shown in Figure 17 was obtained running standard

Figure 17. Standard Curve for the Determination of the Molecular Weight of Protein by Nondenaturing PAGE


Retardation Coefficient
30
10
10,000
30,000
100,000
300,000
500,000
Molecular Weight
Lactalbumin
Carbonic anhydrase
CEA
BSA (monomer)
BSA (dimer)
Urease (dimer)
Urease (tetramer)
CEA: Chicken egg albumin
BSA: Bovine serum albumin

proteins at different % acrylamide gels and the retardation coefficient calculated from the slope of the line resulted from plotting log R_f versus % gel concentration (Figure 18). The samples of PO were run at the same time using 4, 5, 6, 7 and 8% acrylamide gels and the retardation coefficient calculated as described above. The molecular weights of the PO forms are presented in Table 4. A molecular weight value of 210,000 has been reported by Madero and Finne (1982) for shrimp PO. He did not find multiple PO forms. Savagaon and Sreenivasan (1975) have reported molecular weight values of 44,500 and 33,200 for two active PO forms, namely PO_1 and PO_2, respectively, found in Panulirus homarus Linn. They also found a pre-phenoloxidase form with a molecular weight of 44,500 (same molecular weight as PO_1). Ashida (1971) has reported a molecular weight of 40,000 for prephenoloxidase in silkworm. He also reported association of this prephenoloxidase to give a dimer with a molecular weight of 80,000. Such a polymeric association was not investigated in this study.

Figure 18. % Gel Concentration versus Relative Migration of PO Samples and some Protein Standards. The Retardation Coefficient was Calculated from the Slope of each Line



100[log(Rf × 100)]
200
175
150
125
100
2
4
6
8
10
% GEL CONCENTRATION
LACTALBUMIN
BSA MONOMER
TAPO2
EAPO
UREASE DIMER
IPO2
CARBONIC ANHYDRASE
TAPO1

Table 4

Molecular Weights of Several PO Forms Isolated from the Cuticle of Spiny Lobster

PO FORM	MOLECULAR WEIGHT*
IPO1	Not determined
IPO2	189000 ± 14000
EAPO	62500 ± 3000
TAPO1	225000 ± 10000
TAPO2	64000 ± 3500

*Molecular weight ± standard deviation

EFFECT OF BISULFITE ON LOBSTER SHELL PHENOLOXIDASE

Introduction

Sodium bisulfite and other sulfiting agents have been used as food and drug ingredients for many years. They are currently used in a wide variety of foods and food products to inhibit various chemical reactions including enzymatic browning; nonenzymatic browning; to inhibit and control microorganisms; as antioxidants and reducing agents; as bleaching agents; as processing aids; and several secondary uses such as pH control and stabilization.

In the seafood industry, sodium bisulfite is used to prevent black spot (melanosis) in shrimp and lobsters. Recently the use of bisulfite has been of great concern because of its ability to induce asthma and other adverse reactions in certain sensitive individuals. Although most of the problems reported are related to consumption of fruits and vegetables from salad bars, the regulations set by the government (Food and Drug Administration) include shrimp which cannot contain more than 100 ppm as SO_2 in the edible part of the product. Because of these problems, other compounds such as ascorbic acid, sulfur compounds, EDTA and polyphosphates among others have been tested as possible alternatives. To understand the mechanism by which sulfiting agents inhibit melanosis may aid in developing other alternatives as well as providing important basic information on this mechanism.

Materials and Methods

Effect of Bisulfite on Substrate (DL-DOPA)

A spectrophotometric scan from 220 to 300 nm was run on a DL-DOPA mixture containing 0.2 ml of 5 mM DL-DOPA plus 2.7 ml of distilled water, using an LKB spectrophotometer linked to an Apple IIe computer. A similar scan was carried out on a solution containing 0.1 ml of 0.02 M aqueous bisulfite solution added to 0.2 ml of a 5 mM DL-DOPA solution plus 2.6 ml water. Finally, a solution containing 0.2 ml of a 5 mM DL-DOPA was incubated with 0.1 ml of a 0.066 M bisulfite solution for 15 min, and then 2.6 ml of distilled water added and the mixture scanned again. Three complete replicates were conducted.

Trypsin Activity and Bisulfite Concentration

Trypsin activity was determined according to the method of Erlanger et al., 1961. A volume of 2.8 ml of 1 mM BAPNA (N-α-benzoyl-DL-arginine-p-nitroanilide hydrochloride) in 0.05 M Tris-HCl (pH=8.2) containing 0.02 M $CaCl_2.2H_2O$ was mixed with either 0.1 ml of water or 0.1 ml of 0.02 M bisulfite, and 0.1 ml of a trypsin solution (0.4 mg/ml) in a cuvette. The reaction rate was measured at 410 nm for 5 min using a LKB model 4050 spectrophotometer. A similar experiment was carried out incubating 0.1 ml of the trypsin solution with 0.1 ml of 0.066 M bisulfite for 10 min. After this, trypsin activity was determined as before. Trypsin activity was expressed as $\Delta Abs_{410\ nm}$ 10^3/min. Three replicates of the complete experiment were conducted.

Trypsin-Like Activity in a Crude Phenoloxidase Preparation

The procedure of Erlanger et al. (1961) was also used to determine the trypsin-like activity in a crude PO extract with free phenoloxidase activity equaling 6.33 µmoles DL-DOPA per min per ml of crude extract. A volume of 0.2 ml of crude PO extract was used for the determination of "trypsin-like" activity.

Oxygen Uptake of the Phenoloxidase Reaction

A YSI Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co.) was used to monitor the oxygen uptake of the PO reaction. A mixture of 3.0 ml of 5 mM DL-DOPA (pH 6.5) and 0.1 ml of 0.05M phosphate buffer (pH 6.5) was placed in one of the sample chambers. The oxygen probe was inserted into the sample tube and the air expelled through the slot of the plunger. The stirrer in the bottom of the chamber was activated and the meter set to zero with AMP ZERO control. The selector was turned to the AIR position and the instrument was set to 80% saturation with the PROBE control. The system presented an excellent stability (±1% drift) when observed for 5 min. After this, the stirrer was stopped briefly while 0.1 ml of the crude extract was added to the mixture. The stirrer was again activated, the probe reset as explained above, and the percent change in saturation measured against time. Oxygen consumption was also monitored with different concentrations of bisulfite (4.4, 2.2, 1.48, 1.10, 0.88, and 0.74 mM) in the sample mixture.

Effect of Bisulfite on the Intermediate Colored Quinones

A mixture of 2.5 ml of 2.5 mM DL-DOPA and 0.1 ml of an active phenoloxidase extract was allowed to stand until the red color (dopachrome) developed. A 0.066 M bisulfite solution was then added until the mixture was bleached completely. The absorbance of this bleached mixture was very low at 475 nm indicating dopachrome was not present in detectable amounts. The absorption spectrum of the colorless mixture was determined in the range of 250 to 360 nm. Similar scans were carried out for the phenoloxidase preparation, phenoloxidase plus bisulfite, DL-DOPA plus bisulfite, and bisulfite by itself. The scans were done using an LKB Biochrom Ultrospec 4050 spectrophotometer.

A similar experiment was conducted by replacing the bisulfite solution with a 1% ascorbic acid solution. Dilutions of the reaction mixture were done so that the absorbance measured in the range 250-360 nm was not off-scale at any point. Three replicates for each mixture were conducted.

Test for the Detection of Sulfocompounds

Sulfocompounds were detected in the bisulfite-bleached dopachrome mixture as outlined by Feigl (1956) with some modifications. Four drops of the bisulfite-bleached dopachrome mixture were put on a small watch glass and evaporated (without heating) with concentrated hydrochloric acid using nitrogen. A control containing a similar concentration of bisulfite was performed simultaneously. A few drops of thionyl chloride were added and the product treated with two drops of a saturated alcoholic solution of hydroxylamine hydrochloride. The mixture was made alkaline with a 5% sodium carbonate solution. After a few minutes the mixture was acidified with 0.5 N hydrochloric acid. A drop of a dilute solution of ferric chloride was then added. Bisulfite did not respond to this test.

Purification of Cuticle Phenoloxidase

Phenoloxidase was purified by preparative nondenaturing PAGE using a Bio-Rad Model 175 gel tube chamber and a Bio-Rad Model 3000/300 power supply or Model EPS 500/400 (Pharmacia) power supply. Running gel, sample and chamber buffer preparation were performed according to the procedure outlined in the Sigma Technical Bulletin No. MKR-137 (Sigma Chemical Company, 1984). Gel tubes were 14 mm I.D. by 12 cm long, and contained 14 ml of the running gel mixture (5% acrylamide). One milliliter of a crude inert PO extract prepared as outlined earlier was then applied. Gels were run at 8 mA/tube at constant current. Two out of eight tube gels were stained with a 10 mM DL-DOPA solution in 0.05 M phosphate buffer (pH 6.5) to identify PO activity. The same two gels were then stained using a similar solution containing 1% trypsin to develop the inactive PO bands. The remaining six nonstained gels were used to separate the top band (IP01) by slicing off the first 2 mm of the gel and the other inactive PO (IP02) was separated by slicing off at the band 2 mm of gel. The pieces of gel were combined and homogenized in a glass tissue grinder with 10 ml of 0.05 M phosphate buffer (pH 7.2) and the homogenate stirred for 48 hr (to activate PO) using a magnetic stirrer. The homogenate was then centrifuged at 10,000g for 20 min at 4°C and the supernatant containing the activated PO was rerun electrophoretically and extracted as above.

Radiolabeled Bisulfite Treatment

One milliliter of the purified activated PO extract (0.09 µmole DL-DOPA/ml preparation) was mixed by stirring with 5 µl of labeled bisulfite ($NaH^{35}SO_3$, Amersham Corporation) containing approximately 650,000 cpm. After 10 min, 1 ml of "cold" 0.066 M bisulfite solution was added, the mixture was stirred and allowed to stand for 15 min. The mixture was then dialyzed for 72 hr at 4°C against 4 l of deionized double-distilled water and the water changed every 24 hr. A control (no PO extract) was run simultaneously. The dialysis membrane had a molecular weight cut off of 12,000, and was boiled for 30 min in the presence of a small amount of EDTA before use.

Detection of Labeled Bisulfite

Detection of $NaH^{35}SO_3$ was performed by liquid scintillation counting technique using a Beckman, model LS2800 Liquid Scintillation Counter. A volume of 1.5 ml of the dialyzed mixture was mixed with 10 ml of aqueous scintillation liquid (Fisher Scientific Company) in a glass vial and then counted for 10 min.

Phenoloxidase Application to Nitrocellulose Membrane

A square of 2x2 cm of wet nitrocellulose membrane (Schleider and Schuell Inc., Keene, NH) was spotted 20 times with 20 aliquots of 25 µl each (0.5 ml total volume) of the labeled PO preparation. The nitrocellulose membrane was then cut into small pieces and mixed with 10 ml of aqueous scintillation liquid, allowed to stand overnight, and counted as outline above.

Results and Discussion

Effect of Bisulfite on Substrate (DL-DOPA)

The spectrum of DL-DOPA remained unchanged following bisulfite treatment (Figure 19). These results suggested there was no reaction occurring between DL-DOPA and bisulfite. Similar results have been reported by Embs and Markakis (1965) working with vegetable substrates, such as caffeic acid and chlorogenic acid.

Effect of Bisulfite on Trypsin Activity and Trypsin-Like Activity

Bisulfite at the concentrations used appeared to have little effect on trypsin activity. When 0.1 ml of water was substituted for 0.1 ml of 0.02 M bisulfite no significant change in the rate of reaction was observed, even when a mixture of 0.1 ml trypsin solution and 0.1 ml of bisulfite solution was incubated for 10 min (Table 5). However, when 0.066 M bisulfite was used, there was a slight decrease in the reaction rate as shown in Table 5. This decrease could

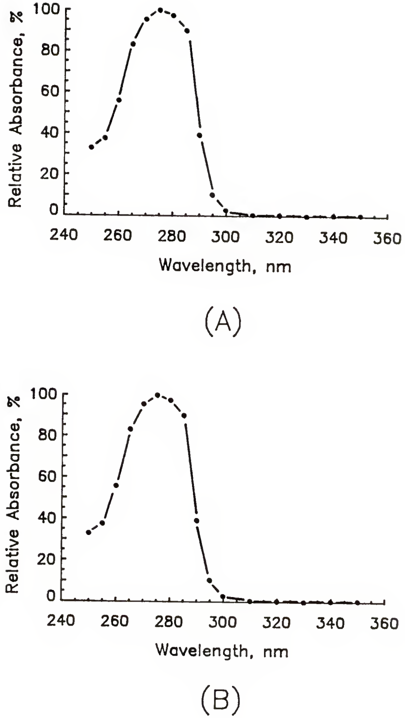


Figure 19. Spectra of DL-DOPA (A) and DL-DOPA/Bisulfite (B) Solutions

Table 5

Trypsin Activity (ΔAbsx10^3/min/0.1 ml Trypsin Solution, 0.4 mg/ml) as Affected by Bisulfite

TREATMENT	TRYPSIN ACTIVITY, ΔAbsx10^3/min
Control	172.8 ± 1.28
0.6 mM bisulfite (Nonincubated)	175.3 ± 1.15
5 mM Bisulfite (Incubated for 10 min)	174.5 ± 0.74
66 mM bisulfite (Incubated for 10 min)	163.7 ± 1.10

be attributed to the disruption of possible disulfide links in the trypsin molecule. No trypsin-like activity was found in the crude extract as determined by the BAPNA method already outlined.

Effect of Bisulfite on the Intermediate Colored Quinones

Initial reaction velocities for the PO reaction were determined spectrophotometrically at 475 nm in the presence of 0, 1.75×10^{-4} M and 3.5×10^{-4} M bisulfite, using 10 mM DL-DOPA (substrate) and a crude PO preparation. Lag phases of 2 and 5 min long were observed at the beginning of the reaction for the lowest and highest bisulfite concentration, respectively. However, the initial reaction rate measured after the lag phase, remained unchanged (Table 6). These results indicated bisulfite under these conditions was not acting as an enzyme inhibitor but it may be chemically reacting with intermediate quinones. Bisulfite may be reducing the quinones to diphenols or it may be forming sulfoquinones by an addition reaction. Increasing the bisulfite concentration to 7.0×10^{-4} M, decreased the phenoloxidase activity by 22% (Table 6). Incubation of the enzyme with this bisulfite solution for 15 and 30 min further inhibited PO by 35 and 45%, respectively (Table 7), indicating inhibition of the enzyme by bisulfite.

Post-addition of bisulfite after forming the chromophore dopachrome resulted instantaneously in a complete disappearance of dopachrome (red color). This red color did not come back even after allowing the mixture to

Table 6

Effect of Various Concentrations of Bisulfite on PO Activity

SODIUM METABISULFITE CONCENTRATION, M	PO ACTIVITY,* µmoles DL-DOPA/min
0.0	0.120 ± 0.004
1.75×10^{-4}	0.119 ± 0.002
3.50×10^{-4}	0.117 ± 0.004
7.00×10^{-4}	0.093 ± 0.004

*Calculated using 5 mM DL-DOPA

Table 7

Effect of Incubating a Crude Trypsin-Activated PO Extract in 7.0×10^{-4} M Bisulfite

INCUBATION TIME, min	PO ACTIVITY, µmoles DL-DOPA/min
0	0.093 ± 0.004
15	0.077 ± 0.001
30	0.064 ± 0.003
45	0.061 ± 0.002
60	0.061 ± 0.001

---

*A lag period of 5 min was observed for all stand for several days at room temperature, indicating an irreversible reaction (bleaching) between bisulfite and dopachrome. Replacing bisulfite with 1% ascorbic acid solution resulted in the complete disappearance of the red color of dopachrome; however, a light red color (dopachrome) was observed in the mixture after storage for 2-3 days at room temperature. It is known that ascorbic acid retards enzymatic browning by reducing the quinones back to diphenols (Whitaker, 1972). Since bisulfite is also a reducing agent, it could be acting in a similar manner to ascorbic acid. But, as pointed out earlier, the possibility also existed that bisulfite could be adding to dopachrome to form sulfoquinones. To test for these possibilities the resulting colorless mixtures were scanned from 250 to 360 nm after the addition of bisulfite and also after the addition of ascorbic acid. The scans are presented in Figure 20. Bisulfite reacted differently with dopachrome compared to ascorbic acid because an extra absorption maximum developed at 340 nm in the sample treated with bisulfite, while in the sample containing ascorbic acid no extra absorption maximum was obtained. This absorption maximum may be due to the formation of a sulfodopachrome leuco compound. The bisulfite-bleached dopachrome mixture gave a positive result (brown color) when tested for sulfocompounds (Feigl, 1956). A control test containing DL-DOPA and bisulfite remained yellow (negative test). Embs and Markakis (1965), Markakis and Embs (1966), and Sayavedra-Soto and Montgomery (1986),

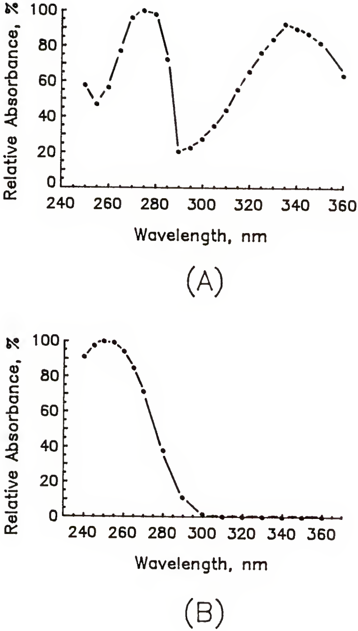


Figure 20. Absorption Spectra of a Metabisulfite-Bleached (A) and an Ascorbic Acid-Bleached (B) Dopachrome Mixture

working with mushroom phenoloxidase and caffeic and chlorogenic acids, have reported the formation of sulfoquinones adducts which absorbed at 290 nm. However, these researchers did not scan at 340-360 nm. Mixtures of p-sulfoquinones and sulfite have been reported to have absorption maximum of 320-345 nm depending on the pH of the mixture (LuValle, 1952). Our results showed that bisulfite was also able to bleach the violet color of melanochrome, an intermediate colored compound formed in the pathway between dopachrome and melanin. Bisulfite did not bleach the dark color of melanin.

According to these results the inhibitory effect of sulfiting agents on the PO system cannot be properly determined by following the dopachrome formation spectrophotometrically. Bisulfite has been reported as a competitive inhibitor by Madero (1982) and Madero and Finne (1982) measuring dopachrome formation at 475 nm. Much of the controversy related to the sulfite inhibition of PO may be due to the use of different assays methods that lead to different observed effects.

Effect of Bisulfite on the Oxygen Uptake of the Phenoloxidase Reaction

A crude active PO extract was used to study the effect of bisulfite on the oxygen uptake of the phenoloxidase catalyzed reaction. Different concentrations of bisulfite ranging from 0.74 to 4.40 mM were used. The results are presented in Table 8.

Table 8

Effect of Bisulfite on Oxygen Uptake by the Phenoloxidase Reaction

TREATMENT	TIME INCREMENTS, succeeding min	CHANGE IN % AIR SATURATION	
		REP 1	REP 2
Buffer + PO extract	10	1	1
DL-DOPA + 4.4 mM bisulfite	15	1	1
DL-DOPA + PO extract	1	8	7
	1	7	7
	1	7	9
	1	8	7
	1	7	8
	1	8	9
4.40 mM bisulfite	1	2	2
	1	0	1
2.20 mM bisulfite	1	7	8
	1	1	1
	1	0	0
1.48 mM bisulfite	1	8	9
	1	3	4
	1	2	3
	1	0	1
1.10 mM bisulfite	1	9	8
	1	7	8
	1	3	3
0.88 mM bisulfite	1	9	7
	1	7	8
	1	5	6
	1	2	3
	1	1	1
0.74 mM bisulfite*	1	1	1
	1	0	0

*PO was incubated with bisulfite for 10 min

A mixture of phosphate buffer plus phenoloxidase, and a mixture of DL-DOPA plus 4.4 mM bisulfite after incubation for 10 and 15 min, respectively, showed very little oxygen uptake. Thus, the amount of oxygen being used by the crude PO preparation was minimal and bisulfite alone did not appear to be reacting with oxygen. It is known that bisulfite is oxidized in aqueous systems by molecular oxygen to yield sulfate (Schroeter, 1966), therefore, one of the possible means by which bisulfite inhibits enzymatic browning is by making oxygen unavailable to the reaction. This may not be an important mechanism of inhibition under the testing conditions, but it may play an important role when bisulfite and oxygen are in contact for a long period of time.

The rate of oxygen uptake for the PO reaction without any bisulfite added was 7.7 % change in air saturation per min. This reaction rate was linear for the first 6 min and then dropped off gradually. In the presence of 0.74 and 0.88 mM bisulfite, the oxygen uptake rate was 8%/min, in the first 2 min, but then progressively dropped to very low levels, eventually reaching 0% (Table 8). At bisulfite concentrations of 1.48 and 2.20 mM, the oxygen uptake was 7 and 8%/min in the first min, respectively, but then eventually dropped off to zero. No enzymatic reaction occurred in the presence of 4.40 mM bisulfite.

Incubation of the enzyme with 1.48 mM bisulfite for 10 min prior to assaying resulted in no oxygen uptake being observed. This indicated that bisulfite also inactivated the enzyme, possibly by inhibiting PO irreversibly. Two reactions may be occurring at the same time; the first reaction being,

$$\text{PO} + \text{DOPA} \text{ ------> Dopachrome } (k_1)$$

and the other,

$$\text{PO} + \text{HSO}_3^- \text{ --------> Inactive PO } (k_2)$$

Under the assay conditions the first reaction appeared to be occurring faster ($k_1 > k_2$) but at higher bisulfite concentrations the inhibition of the PO catalyzed reaction predominated. At the 4.40 mM bisulfite concentration, inhibition was instantaneous (0% oxygen uptake), while at 1.48 mM bisulfite concentration, inhibition occurred after three minutes.

Electrophoresis of Bisulfite-Treated Phenoloxidase

The inhibition of PO by bisulfite appeared to be irreversible. Non-denaturing PAGE of PO crude extracts incubated for 10 min with 0.012 M and 0.066 M bisulfite indicated the absence of PO activity bands or the development of less intense bands (Figures 21c and 21d) relative to a control (Figure 21a) when stained with DL-DOPA. The higher the concentration of bisulfite the more efficient the inhibition. Trypsin-activated PO was also irreversibly inhibited by bisulfite (Figures 21e and 21f). Of the two bands developed in the trypsin-activated PO control (Figure 21b), one was completely inhibited, and the other was partially inhibited, as measured by band intensity

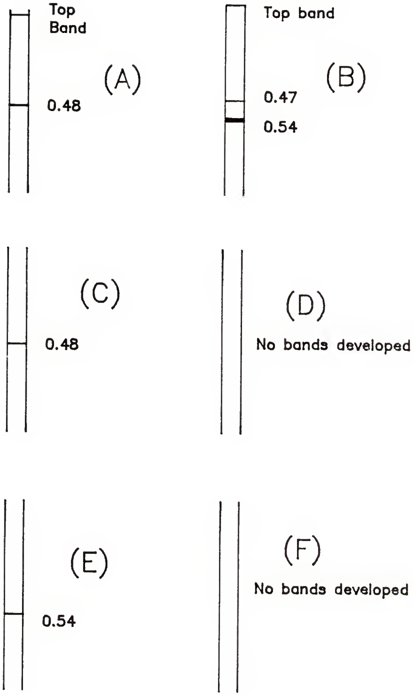


Figure 21. Electrophoresis of EAPO (A), TAPO (B), EAPO in the Presence of 12 mM bisulfite (C), EAPO in the Presence of 66 mM bisulfite (D), TAPO in the Presence of 12 mM Bisulfite (E), and TAPO in the Presence of 66 mM Bisulfite (F)

(Figure 21e and 21f). Similar results have been reported by Sayavedra-Soto and Montgomery (1986) working with mushroom and pear PO and caffeic and chlorogenic acids as substrates.

Mode of Action of Bisulfite on Phenoloxidase

The results of the above experiments have shown the inhibitory nature of bisulfite on lobster cuticle PO. One possible explanation for this inhibition may be by disruption of disulfide bonds present in the PO molecule. Experiments using dialysis, nitrocellulose membranes and radioactive labeling techniques were conducted to test this explanation. The purified EAPO preparation obtained by performing preparative electrophoresis twice as outlined earlier (Materials and Methods) was used for these experiments. The IPO1 (top band) and IPO2 (R_f = 0.32) bands were separated from the first electrophoretic gels. The pieces of gel containing the two PO forms were pooled and homogenized with 10 ml of 0.05 M phosphate buffer (pH 7.2) and stirred for 48 hr at $4^{o}C$ using a magnetic stirrer. The pieces of gel were then separated by centrifugation and the supernatant rerun electrophoretically. Only one active PO form (EAPO) developed (Rf = 0.40) as demonstrated by electrophoresis .

Dialysis and of a Labeled Bisulfite/PO Mixture

A volume of 1 ml of the purified active PO preparation was treated with 5 µl of $NaH^{35}SO_3$ containing 650,000 cpm and 1 ml of "cold" bisulfite and the mixture dialyzed against deionized and distilled water. Total concentration of initial bisulfite was 0.066 M. The level of ^{35}S in the dialyzed mixture was detected by liquid scintillation counting. The results presented in Table 9 showed that most of the labeled bisulfite migrated out through the dialysis membrane, indicating no detection of bisulfite binding to the EAPO form, although the counts in the sample containing the enzyme were slightly higher compared to the control.

A volume of 2 ml of the dialyzed sample mixture was treated with acetone (2 ml) to precipitate the protein. The cloudy mixture obtained was centrifuged at 30,000g for 30 min at 4° C. The protein precipitate was dissolved in 1 ml of 0.05 M phosphate buffer (pH 7.2) and the solution tested for labeled bisulfite using the liquid scintillation counter. The supernatant was also checked for labeled bisulfite. Practically all the labeled bisulfite was found in the supernatant. Very little ^{35}S was found in the protein precipitate (Table 10), thus confirming no binding of the labeled bisulfite to the PO.

Labeling Assays Using Nitrocellulose Membranes

The mixture of PO, labeled bisulfite and "cold" bisulfite (as prepared for dialysis) was used to spot the nitrocellulose membrane which is known to bind proteins. Twenty aliquots of 25 µl each of the above mixture were applied on a piece (2x2 cm) of nitrocellulose membrane wet with 0.05 M phosphate buffer (pH 7.2). After 30 min the nitrocellulose membrane was rinsed five times with water, cut into small pieces, and mixed with 10 ml of aqueous

Table 9

Counts per Minute* in the Dialyzed Mixture as Detected by Liquid Scintillation Counter

Control	Sample
263	517
266	507

*Sample size was 1.5 ml

Table 10

Counts per Minute* in Supernatant and Precipitate after Treatment of the Dialyzed Mixture with Acetone

	Supernatant	Precipitate
Control	147	72
	138	68
Sample	273	87
	285	79

*Sample size was 1.0 ml

scintillation liquid. The mixture was allowed to stand overnight and then counted for 10 min. A control was run under similar conditions but the volume of the PO preparation was replaced with phosphate buffer only. The number of cpm (counts per minute) obtained for the sample and control were under 100, which was considered to be background reading. Thus, it was not possible to detect any covalent binding of labeled bisulfite to PO under these conditions. These results are very preliminary, since the minimal mole ratio (moles of disulfide bonds/mole of protein) that could be detected was not determined.

SEASONAL VARIATION OF LOBSTER SHELL PHENOLOXIDASE

Introduction

For crustacea to grow, they must shed their exoskeleton. The new exoskeleton is bigger and the animal grows (swells) to fill it. The process is called molting and involves a series of complex biochemical, physiological and morphological changes. Phenoloxidase (PO) is thought to be responsible for the sclerotization (hardening) of the shell after molting (Cobb, 1977). In a study done by Koburger et al. (1985), melanosis developed in thawed lobster tails stored on ice at 4°C. An intense blackening of the epidermis occurred with the dark pigment migrating to the meat. In some lobsters, the appearance of melanosis was obvious after 2 days of storage, while other lobsters took up to 5 days to blacken. It appeared that phenoloxidase was located in the shell or epidermis of the animal and that the levels of phenoloxidase activity varied from lobster to lobster. This variation was attributed to molting stage. Preliminary studies indicated the presence of phenoloxidase in the shell (cuticle) of the animal and very little phenoloxidase activity in the lobster flesh. Studies were carried out to determine the relationship between phenoloxidase levels in lobster cuticle and seasonal variations as well as molting stage.

Materials and Methods

Florida lobsters were obtained from Whitney Marine Laboratory at Marineland, FL. The animals were maintained in tanks with flow-through sea water circulation. Water temperature was not controlled, and varied according to the season. Every month, during the 1985-86 season, 2-3 lobsters were picked at random for phenoloxidase analysis; a total of 26 lobsters were analyzed for seasonal variation of PO levels. Phenoloxidase levels were also analyzed in lobsters (24 specimens) at different molting stages: molt (newly molted lobsters), postmolt, intermolt and premolt as described by Stevenson (1985). The different molting stages were determined by morphological changes in the epidermis, by the hardness of the exoskeleton, by following the development of setae in the pleopods and also by the appearance of an ecdysial line along the branchiostegites, which is characteristic of a premolt lobster (Travis, 1954).

Lobster tails were kept frozen and transported in ice from Marineland to Gainesville (a 1.75-hr trip). Only tail shell was used for the PO determination. Pleopods and epidermis were not included in the analysis.

Phenoloxidase Extraction

A crude PO extract was prepared by homogenizing in a blender 1 part (by weight) lobster shell with 4 parts (by volume) 0.05 M potassium phosphate buffer (pH 7.2) for 2 min (four blasts of 30 sec each) at 4°C. The homogenate was centrifuged in a Sorvall RC-5 Superspeed Refrigerated Centrifuge at 10,000g for 20 min at 4°C. The supernatant (crude extract) was filtered through a Whatman # 1 filter paper and stored at -20°C until assayed.

Protein Determination

Protein content of the crude extract was determined using the biuret method (Clark and Switzer, 1977) where 1 ml of the crude extract was placed in a test tube and 4 ml of biuret reagent added, vortexed for a few seconds, and let stand for 30 min at room temperature. The absorbance was then measured at 540 nm using a Gilford Instruments spectrophotometer Model 250. Bovine serum albumin (BSA) was used as the standard protein. Three replicates were conducted.

Determination of Phenoloxidase Activity

Phenoloxidase activity was determined by mixing 0.1 ml of the crude enzymatic preparation with 2.8 ml of 5 mM DL-β-3,4-dihydroxyphenylalanine (DL-DOPA) as substrate in 0.05 M phosphate buffer (pH 6.5) with and without trypsin at 25°C (Savagaon and Sreenivasan, 1978). An LKB Model 4050 Ultrospec spectrophotometer linked to an Apple IIe computer was used to determine the initial rate of increase in absorbance at 475 nm. Phenoloxidase activity was defined as the µmoles of DL-DOPA transformed per min under the testing conditions (e.g. a given volume of PO preparation, 23°C and total reaction volume of 3 ml).

Electrophoresis Analysis

Analytical nondenaturing polyacrylamide gel electrophoresis (PAGE) was used to test for PO occurrence at molt, postmolt, intermolt, early premolt, and late stage premolt. Cast-off exoskeletons were also analyzed for PO. A Bio-Rad Model 3000/300 or Pharmacia power supply was used in the electrophoresis. Gel tubes 0.5 cm I.D. by 12.5 cm high, containing 5 % acrylamide were run at constant current of 3 mA/tube. Sample size was 0.1 ml (0.2 ml used for molt stage lobster samples) and chamber buffer was 0.01 M tris (hydroxymethyl) aminomethane/0.04 M glycine, pH 8.3. The electrophoretic procedure utilized is that outlined in the Technical Bulletin No. MKR-137 (Sigma Chemical Company, 1984). Gels were specifically stained for PO activity using a 10 mM DL-DOPA solution with or without trypsin.

Results and Discussion

Seasonal Study

There was a wide variation in the specific activity of PO in the shell of the lobsters tested (Table 11). Addition of trypsin to the PO crude extract increased the PO activity from 2-fold to over 120-fold in some cases. Values ranged from 0.01 to 0.80 and from 0.32 to 37.00 µmoles of DL-DOPA min^{-1} mg protein^{-1} for free active and trypsin-activated PO's, respectively (Table 11). An increase in the levels of trypsin-activated PO (inert PO or prophenoloxidase) was observed during the period from May to September (Figure 22), which were the warmest months of the year.

Table 11

Free PO and Trypsin-Activated PO (TAPO) Specific Activity in Cuticle Extracts of Lobsters Collected During the 1985-86 Season

SAMPLE	TAPO ACT. EU/ml**	FREE PO ACT. EU/ml	PROTEIN CONTENT mg/ml	SP. ACT TAPO EU/mg	SP. ACT* FREE PO EU/mg
10-01-85	0.35	0.15	0.50	0.70	0.30
10-08-85	2.37	0.17	0.84	2.82	0.20
10-21-85	0.30	0.05	0.64	0.47	0.08
10-29-85	0.54	0.14	0.66	0.82	0.21
01-08-86	0.59	0.04	0.31	1.90	0.13
01-20-86	0.87	0.05	0.28	3.11	0.18
01-21-86	0.17	0.03	0.28	0.61	0.11
10-22-85	0.07	0.01	0.19	0.37	0.05
10-23-85	0.14	0.03	0.24	0.58	0.13
12-12-85	0.94	0.05	0.40	2.35	0.13
12-16-85	0.27	0.03	0.40	0.68	0.08
04-19-86A	3.32	0.03	0.40	8.30	0.08
04-19-86B	0.49	0.02	0.32	1.53	0.06
04-19-86C	3.70	0.05	0.48	7.71	0.10
05-15-86A	7.54	0.06	0.40	18.85	0.15
05-15-86B	8.88	0.19	0.24	37.00	0.79
05-15-86C	2.80	0.05	0.33	8.48	0.15
06-16-86A	0.06	0.002	0.19	0.32	0.01
06-16-86B	0.17	0.02	0.28	0.61	0.07
06-16-86C	0.11	0.02	0.30	0.37	0.07
06-16-86D	0.11	0.02	0.21	0.52	0.10
08-20-86A	10.77	0.09	0.46	23.41	0.20
08-20-86B	7.41	0.06	0.41	18.07	0.15
09-16-86A	6.72	0.06	0.38	17.68	0.16
09-16-86B	2.22	0.04	0.36	6.17	0.11

*Phenoloxidase activity

**EU (enzyme units) are expressed in μmoles DL-DOPA/min

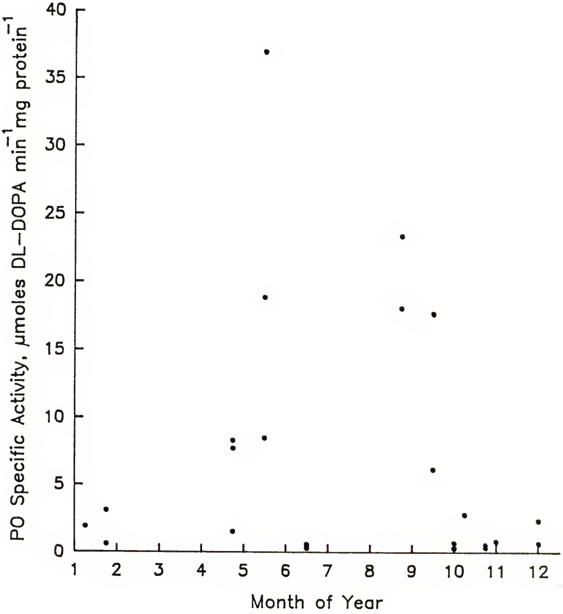


Figure 22. Trypsin-Activated PO versus Month of the Year (1985-86 Season)

During the course of this study, it was observed that little molting occurred during the cold months while most molting took place in the summer. A relationship between molting and water temperature has been observed by Travis (1954). She found that the molting process is directly correlated with water temperature. Thus, a relationship between molting cycle and PO levels could exist. It is known that PO is involved in the tanning of cuticle (in the case of a molting animal, the newly forming cuticle) by catalyzing the formation of quinones which form cross-links with cuticular proteins (Stevenson, 1985; Andersen, 1979). For PO to catalyze the production of quinones, it must be in an active state. Therefore, the levels of free PO activity (endogenously activated PO or EAPO) should be higher during the warm months (May-September period) when lobsters molt more frequently. However, this was not the case as shown in Figure 23. Unlike inert PO, there was no increase in free PO activity during the May-September period. This was contradictory because the prophenoloxidase must become activated before it can efficiently act on the substrate (see Chapter 1).

Relationship Between Molting Stage and Phenoloxidase Levels

A closer evaluation of Figure 23 revealed that there was one lobster which had a high free PO activity during the sampling from May-September. This lobster was in the premolt stage, as determined by the presence of the new cuticle beneath the old one. The new cuticle looked like

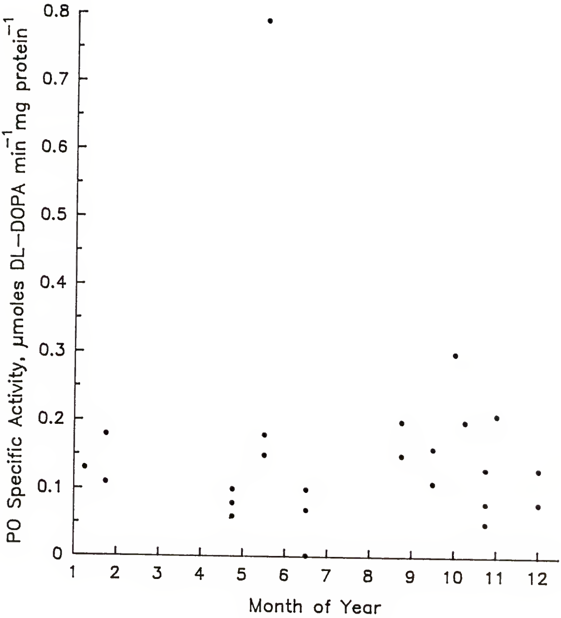


Figure 23. Free PO Activity versus Month of the Year (1985-86 Season)

the old one (same color and spots), but was very thin, uncalcified and very fragile, having no hardness. The part of the cuticle formed at this stage may have been the epicuticle. It was hypothesized that inert PO's (IPO1 and IPO2) were being activated at this premolt stage in order to provide the cross-linking agents (quinones) for the cuticle sclerotization (tanning). To confirm these results, several other lobsters at the premolt stage were selected and analyzed for trypsin-activated (inert PO's or prophenoloxidases) and free PO activity as previously described. The premolt lobsters were identified by looking for the development of the setae on pleopods under the dissecting microscope, by the state of development of the epidermis, and by the appearance of an ecdysial line along the branchiostegites. Such a line becomes visible 3-4 days or 2-3 weeks before ecdysis in the summer months and cold months, respectively (Travis, 1954). The lengthening of the premolt stage in the winter months may be due to a decrease in the rate of physiological activities associated with molting.

Lobsters in molt, postmolt, and intermolt stages as well as cast-off exoskeletons (exuviae) were also analyzed. Molt lobsters were newly molted lobsters (lobsters within one day of molting) having a very soft exoskeleton. Postmolt and intermolt lobsters were identified mainly by looking at the epidermis. At these stages the epidermis is bound to the cuticle and intermolt lobsters present a harder exoskeleton. The epidermis detaches from the cuticle at the beginning of premolt (early premolt stage) in a process known as apolysis (Jenkin and Hinton, 1966) and secretes molting fluid containing digestive enzymes to break down the inner layers of the old cuticle.

Phenoloxidase activities for lobsters at different molting stages are presented in Table 12. An activation of phenoloxidase in premolt lobsters, especially in the late premolt stage was observed. Free and trypsin-activated PO activity were identical at the late premolt stage, suggesting the occurrence of a fully activated phenoloxidase. This was confirmed by analysis of the exuvia of newly molted lobsters where addition of trypsin to the PO extracts did not affect the PO activity levels, indicating the existence of a fully active PO. However, lobsters in early premolt and other stages did not present a fully activated prophenoloxidase (Table 12).

The data in Table 12 were statistically analyzed using Duncan's Multiple Range test. The data were divided into two classes, namely treatments (with two levels: trypsin-treated and untreated) and stages (with five levels: molt or newly molted lobsters, postmolt, intermolt, early premolt and late premolt). The statistical analysis showed significant differences ($P<0.01$) between treatments and stages. Also a significant interaction ($P<0.01$) between the two classes occurred indicating that the differences between the two treatments (i.e. the differences between free and

Table 12

Free PO, Trypsin-Activated PO Levels and Protein Content in Cuticle Extracts from Lobsters at Various Molting Stages

	TAPO	FREE PO	PROTEIN CONTENT	TAPO	FREE PO*
SAMPLE	EU/ml**	EU/ml	mg/ml	EU/mg	EU/mg
Newly molted 1	0.11	0.02	0.21	0.52	0.10
Newly Molted 2	0.06	0.04	0.19	0.32	0.21
Newly molted 3	0.07	0.01	0.20	0.35	0.05
Newly molted 4	0.05	0.04	0.23	0.22	0.17
Newly molted 5	0.08	0.02	0.21	0.38	0.10
Newly molted 6	0.23	0.01	0.55	0.42	0.02
Postmolt 1	0.47	0.02	0.33	1.42	0.06
Postmolt 2	0.38	0.05	0.35	1.09	0.14
Postmolt 3	0.21	0.02	0.38	0.55	0.05
Postmolt 4	0.07	0.03	0.27	0.26	0.11
Postmolt 5	0.10	0.01	0.25	0.40	0.04
Postmolt 6	0.08	0.03	0.20	0.40	0.15
Intermolt 1	1.98	0.01	0.43	4.60	0.02
Intermolt 2	5.14	0.04	0.39	13.18	0.10
Intermolt 3	3.27	0.10	0.26	12.57	0.38
Early premolt 1	3.49	0.12	0.13	26.85	0.92
Early premolt 2	7.60	0.09	0.24	31.67	0.38
Early premolt 3	3.30	0.04	0.11	30.00	0.36
Early premolt 4	7.67	0.09	0.26	29.50	0.35
Early premolt 5	5.52	0.10	0.33	16.72	0.30
Early premolt 6	5.31	0.11	0.27	19.67	0.41
Late premolt 1	1.82	2.05	0.30	6.07	6.83
Late premolt 2	0.96	1.24	0.14	6.86	8.86
Late premolt 3	0.93	0.88	0.18	5.17	4.89

*Specific activity, EU/mg protein

**EU (enzyme units) are expressed as µmoles DL-DOPA/min trypsin-activated PO levels) depended on the particular molting stage of the lobster. Table 13 summarizes the statistical analysis for the interaction effect.

Newly molted lobsters did not have high levels of either active or trypsin activated phenoloxidase in the shell (Table 13). The statistical analysis showed no significant difference ($P>0.05$) among the means for free PO activity for the stages molt, postmolt, intermolt and early premolt. There was also no significant difference ($P>0.05$) between the two treatments for the molt, postmolt and late premolt lobsters, but there was a significant difference ($P<0.05$) among the two treatments, intermolt and early premolt.

The data are better depicted in Figure 24. The free PO curve showed a constant level from the molt to early premolt stages, and increased at late premolt. The trypsin-activated curve demonstrated low levels of PO activity during molt and postmolt. After this stage, trypsin-activated PO levels started increasing sharply and then dropped off by late premolt; not being significantly different ($P>0.05$) from free PO at late premolt. These results suggested an increase in PO as inactive forms (IPO1 and IPO2) from molt to early premolt that then became activated at late premolt.

The shell of newly molted lobsters was relatively soft and flexible since the different layers of the cuticle have not yet completely formed. Since one of the known functions

Table 13

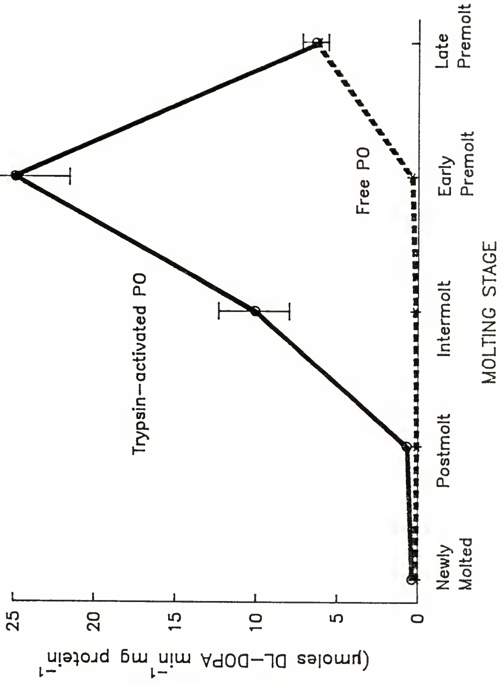
PO Specific Activity in the Cuticle of Lobsters at Different Molting Stages*

MOLTING STAGE	NO TRYPSIN	TRYPSIN
Newly molted	0.11 ± 0.07 C**	0.37 ± 0.10 C
Postmolt	0.09 ± 0.05 C	0.69 ± 0.46 C
Intermolt	0.17 ± 0.19 C	10.12 ± 4.79 B
Early premolt	0.45 ± 0.23 C	25.73 ± 6.11 A
Late premolt	6.86 ± 1.99 B	6.03 ± 0.85 B

*Average ± standard deviation

**Means with the same letter in the same row or column are not significantly different from each other ($P<0.05$)

Figure 24. Free and Trypsin-Activated PO Levels at Various Molting Stages (Bars Represent Means ± Standard Deviation for the Trypsin-Activated PO Line)


PO Specific Activity
(μmoles DL–DOPA min^{-1} mg protein^{-1})
0
5
10
15
20
25
Trypsin–activated PO
Free PO
Newly Molted
Postmolt
Intermolt
Early Premolt
Late Premolt
MOLTING STAGE

of PO is to produce the tanning quinones, partial sclerotization of the epicuticle and preecdysial procuticle may have occurred before molting (i.e. at the premolt stage) when active PO levels were high, and probably the quinone levels increased. Due to the antiseptic properties of quinones, this apparent increase in their levels may help in preventing the penetration of microorganism through the unfinished cuticle.

It has been reported (Stevenson, 1985) that after ecdysis (postmolt), the principal layer is secreted, and hardening of the cuticle begins by calcification and sclerotization with the maximum strength of the cuticle being achieved in the intermolt stage. A relationship between molting and the build up of melanin precursor in the premolt stage has been reported in shrimp (Cobb, 1977). However, sclerotization does not always mean discoloration. Ortho-diphenol N-acetyldopamine (NADA) has been reported as the cross-linking agent in insects (Andersen, 1979) and it is also believed to act in the sclerotization of the crustacean cuticle (Stevenson, 1985). This compound is the substrate for the PO producing NADA-quinone which has been reported to react spontaneously with free amino groups in the cuticular proteins (Andersen, 1979).

High levels of phenoloxidase specific activity were found in the cast-off cuticle or exuvia (Table 14). Phenoloxidase in the three exuviae analyzed was fully activated as indicated by the values obtained in the presence and absence of trypsin. These results confirmed that PO was fully activated in the late premolt stage, probably a few days before molting.

The high values of PO activity found in the exuviae were not the result of large PO activity but instead they were the result of a very low protein content of the cuticle (Table 14). Probably most of the nutrients, especially proteins, in the old cuticle (cuticle to be shed-off) were digested and reabsorbed by the lobster. It has been reported that the cuticle functions as a food storage reservoir (Stevenson, 1985). This may explain the low levels of protein found in the cast-off cuticle.

Most reports dealing with PO levels as related to molting have been associated with blood phenoloxidase. Summers (1967) reported the lowest levels of blood phenoloxidase in postmolt and the highest during intermolt in the fiddler crab. He observed a reduction of blood phenoloxidase activity during premolt. He speculated that the decrease in blood PO levels during intermolt could be due to accumulation of PO in the cuticle of the crab in preparation for the molting event.

Phenoloxidase Forms as Related to Molting Stage

Nondenaturing polyacrylamide gel electrophoresis (PAGE) conducted on crude extracts from lobster shells at the various molting stages showed the occurrence of different PO forms (Figures 25 and 26). At all molting stages active PO forms could be detected after staining the gels with 10 mM

Table 14

Free PO, Trypsin-Activated PO Levels and Protein Content in Lobster Exuvia Extracts

	TAPO	FREE PO	PROTEIN CONTENT	TAPO	FREE PO*
SAMPLE	EU/ml**	EU/ml	mg/ml	EU/mg	EU/mg
Exoskeleton 1	0.40	0.40	0.05	8.00	8.00
Exoskeleton 2	0.34	0.31	0.07	4.81	4.43
Exoskeleton 3	0.38	0.37	0.09	4.22	4.11

*Specific activity, EU/mg protein

**EU (enzyme units) are expressed as µmoles DL-DOPA/min

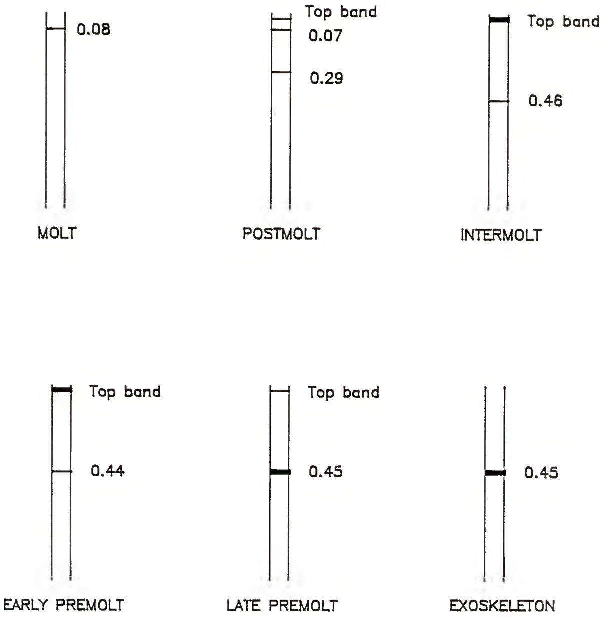


Figure 25. Electrophoresis of Cuticle PO Extracts at Various Molting Stages (DOPA Stained Gels). The Numbers by the Band Represent the R_f

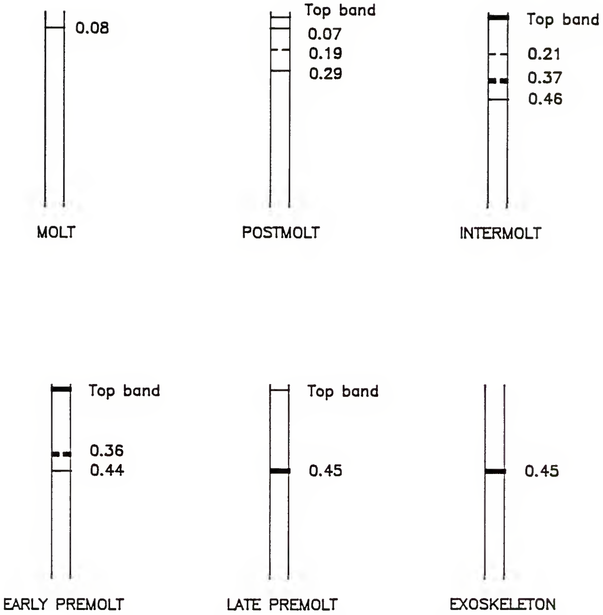


Figure 26. Electrophoresis of Cuticle PO Extracts at Various Molting Stages (DOPA/Trypsin Stained Gels). Interrupted Lines Indicate Inert PO Forms Activated by Trypsin. The Numbers by the Band Represent the R_f

DL-DOPA (pH 6.5); however, PO activity was lower at the molt, postmolt, intermolt, and early premolt stages than at late premolt, as judged by the intensity of the PO bands (Figure 25). Inactive PO forms were also present at some molting stages. They were detected by staining the gels with 10 mM DL-DOPA (pH 6.5) containing trypsin. Three inactive PO forms were present as shown in Figure 26 (interrupted bands and top band). The most dominant inactive PO forms (most intense PO bands) developed at intermolt and early premolt stages. No inactive PO forms were found at molt and late premolt stages. Electrophoretic analysis of the cast-off exoskeleton and crude extracts from shells of late premolt animals showed the occurrence of a single active PO band (Figure 25). No inert PO forms were found in the cast-off exoskeleton indicating a total transformation of the inert PO's to one single active PO form. This PO form is probably responsible for the increase in free PO activity at late premolt stage and for the production of the cross-linking quinones for sclerotization of the cuticle. Thus the activation of the inert PO in vivo in the spiny lobster appeared to be related to the molting cycle of the lobster.

CONCLUSIONS

Phenoloxidase (PO) is found in spiny lobster shell as inert enzymes (IPO1 and IPO2) that are activated by trypsin producing two active PO forms (TAPO1 and TAPO2) with up to 120-fold increased PO activity. Other serine proteases such as elastase and chymotrypsin do not activate PO. Triton X-100 and repeated freezing and thawing do not activate the enzyme. The inert PO's become active during storage at 4°C producing only one active PO form (EAPO). This activation appears to be triggered by an endogenous protease. The mode of action of this protease appears to be somewhat different than trypsin as demonstrated by kinetic and electrophoretic data.

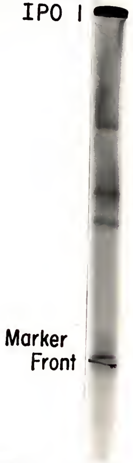
Sodium bisulfite inhibits the PO reaction by reacting with the intermediate quinones probably forming sulfoquinones and by irreversibly inhibiting PO. The nature of this inhibition is still not clear. It appears bisulfite does not bind to PO.

The activation of PO *in vivo* appears to be related to the molting cycle of the lobster. High levels of active PO (EAPO) are found in lobsters ready to molt (late premolt stage) and cast-off cuticles, while high levels of inert PO (IPO1 and IPO2) and low levels of active PO are found in lobsters at intermolt and early premolt stages. Low levels of both inert and active PO's are found in newly molted and postmolt lobsters. It appears PO levels accumulate as inert forms which become activated when needed for hardening (tanning or sclerotization) of the new cuticle or any other function associated with phenoloxidase utilization.

APPENDIX

Figure A-1. Electrophoresis of a 30-50% Ammonium Sulfate Fraction (nonactivated) Using DL-DOPA (A) and DL-DOPA/Trypsin (B) as Specific Enzyme Staining Agents

(A)



(B)

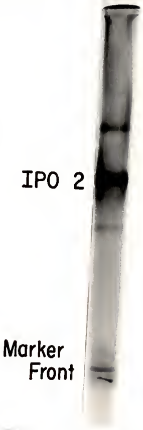


Figure A-2. Electrophoresis of a Trypsin-Activated (A) and an Endogenously Activated (B) PO Crude Extract

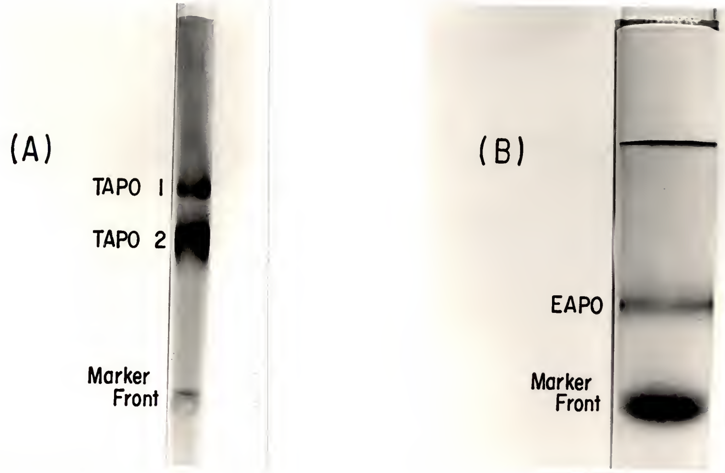
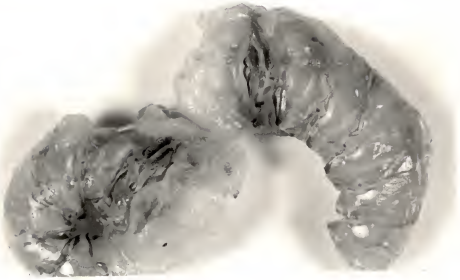
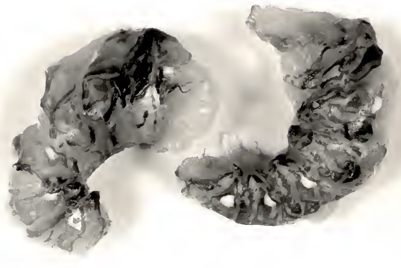

(A)
TAPO 1
TAPO 2
Marker Front
(B)
EAPO
Marker Front

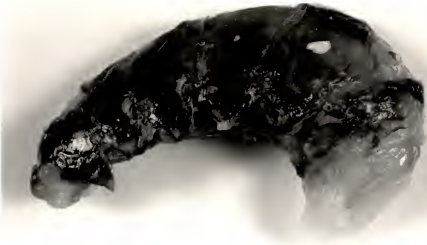
Figure A-3. Different Developmental Stages in the Formation of Lobster Cuticle: No New Cuticle but Epidermis Detached from Old Cuticle (A), Initial Signs of the Newly Forming Cuticle (B), Advanced Signs of the Newly Forming Cuticle (C), and Newly Forming Cuticle Almost Completely Formed as in a Late Premolt Lobster (D)



(A)



(B)



(C)

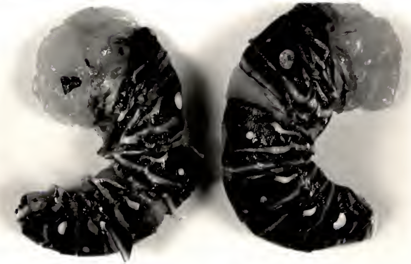


Figure A-3--continued (D)

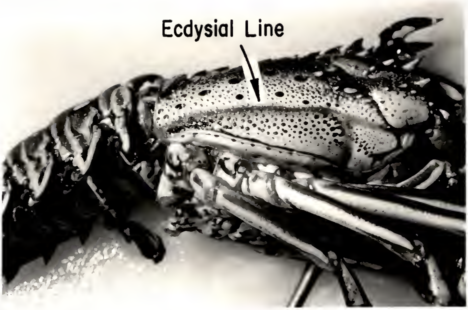


Figure A-4. Ecdysial Line that Appears along the Branchiostegites in a Premolt Lobster

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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